(Partial) Globozoospermia

an allround study
on what is not all round

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ISBN
978-90-9028528-3

Cover design and lay-out
Promotie In Zicht, Arnhem

Painting cover
'*Fernando Pessoa e sua ilha*', Pirandello, Goiania, Brazilië

Printing
Ipskamp Drukkers BV, Enschede

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Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. Th.L.M. Engelen,
volgens besluit van het college van decanen
in het openbaar te verdedigen op

donderdag 20 november 2014
om 10.30 uur precies

door

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"For small erections may be finished by their first architects; grand ones, true ones, ever leave the copestone to posterity. God keep me from ever completing anything. This whole book is but a draught—nay, but the draught of a draught. Oh, Time, Strength, Cash, and Patience!"

Herman Melville , Moby-Dick; or, The Whale (1851)

Voor Felix en co.

LIST OF ABBREVATIONS

ANOVA	analysis of variance
AR	androgen receptor
ART	assisted reproductive techniques
CKIA	computerised karyometric image analysis
CMA3	chromomycine A3
DET	double embryo transfer
DAZ	Deleted in Azoospermia
DAZLA	Deleted in Azoospermia Like Autosomal
DNA	deoxyribonucleic acid
FISH	fluorescence in-situ hybridization
hCG	human choriongonadotrofine
HST	hypo- osmotic swelling test
ICSI	intra cytoplasmic sperm injection
IUI	intra uterine insemination
IVF	in vitro fertilisation
KM-2	histone modification that recognizes H4 acetylated at lysine 8, 12 and 16
OAT	oligoasthenoteratospermia
OPU	ovum pick-up
P1	protamine 1
P2	protamine 2
SCSA	sperm chromatin structure assay
SET	single-embryo transfer
SNP	single nucleotide polymorphism
TEM	transmission electron microscopy
TH2A	testis-specific histon 2A
TH2B	testis-specific histon 2B
TUNEL	TdT (terminal deoxynucleotidyl transferase)-mediated dUTP-biotin nick-end labelling
VCM	volume concentration motility count, without processing
WHO	World Health Organization

CONTENTS

Chapter 1

Introduction

Genetics of male infertility

Male infertility involves a heterozygous group of conditions that either consists of an abnormality in sperm count (oligozoospermia), motility (asthenozoospermia), morphology (teratozoospermia) or a combination of these three factors. Although couples affected by male infertility can be treated by Intra Cytoplasmic Sperm Injection (ICSI), which was introduced in 1992 [1], the diminished production or poor quality of sperm cannot be cured yet. The impact of the male factor in infertility is substantial: for more than 50% of the infertile couples, sperm production is impaired. Nowadays in the western world, about 60 % in vitro treatments consist of ICSI, achieving similar and sometimes even better results than conventional IVF [2]. Although ICSI has proven to be an effective technique to solve the poor fertility capacity of the sperm, there are still some concerns about the safety of this treatment, specially the long-term risks. There is some evidence of an increased risk of minor and major congenital malformations in ICSI children [3, 4]. The major malformation rate varies between 0.7 and 9.1 % in comparison to 0.5- 7.2 % in naturally conceived children [4-7]. While the follow-up studies on ICSI children are reassuring up to adolescence [8], the risk towards late-onset disease manifestations is unknown. In addition, it is still unclear whether the prevalence of male infertility is affected by forcing fertilization by ICSI, which would allow the propagation of possible underlying genetic defects to the next generation. Although not all cases of male infertility have a genetic origin, the massive implementation of ICSI may lead to accumulation of mutations that would otherwise have disappeared from the population. Interestingly, after the successful introduction of ICSI, many clinicians and researchers doubted about the need of continuing andrologic research: where male infertility cannot be solved, the presence of sperm in ejaculates, epididymis or testis is sufficient to treat male infertility. With the advance of technology many investigators, including ourselves, have focused to find a genetic cause for OAT (defined as $<1 * 10^6$ spermatozoa/ ml) other than the already few known. Known causes are numerical chromosomal abnormalities such as Klinefelter (XXY) and Robertsonion translocations, which account for up to 10% in severe OAT or azoospermia. DNA microdeletions in the Y chromosome, e.g. in the *AZF* region are responsible for 5-15% of oligozoospermic men [9]. A recessive *CFTR* mutation can cause azoospermia by a congenital bilateral absence of the vas deferens (CBAVD) and accounts for 1-2 % of the subfertility cases[9].

(Re-)search for genetic causes

In the search for genes responsible for male infertility, our group started a search for genetic mutations. Until now, our research group has used two methods to perform this search.

Screening for genetic aberrations in known genetic factors in male infertility

In order to find mutations in known genes that are involved in spermatogenesis, our Radboudumc research group screened groups of subfertile men, mostly ICSI-patients [10]. We screened the *DAZLA* gene (Deleted in Azoospermia Like Autosomal), that is proven to be involved in spermatogenesis in mice [11] and testis specifically expressed, as well as homologous to *DAZ* (Deleted in Azoospermia), that is found to be deleted in some cases of severe oligozoospermia and azoospermia [12]. Unfortunately we found no mutations in 44 subfertile men. We then focused on increased length of the CAG repeats in the androgen receptor (AR), which might be associated with male subfertility. However, analysis of the *AR* gene in 75 subfertile men, ranging from azoospermia to severe OAT, did not reveal an association between the CAG repeat length and subfertility in our cohort [10].

Familial male fertility

The methodology used by our group in Radboudumc prior to the start of this thesis was to locate and investigate families in which several members were affected with male infertility. During this research we managed to establish that male infertility occurs more often in brothers and maternal uncles, suggesting either X-linked or autosomal dominant inheritance with sex-limited expression of the phenotype [13]u. In fact, linkage analysis in a Dutch family with multiple subfertile males provided evidence for sex-limited expression of an autosomal trait [14]. X-linked inheritance was excluded in this family consisting of six siblings and a maternal uncle that suffered from azoospermia by genotyping with X-chromosomal markers and by performing X-chromosome-specific array Comparative Genomic Hybridization (ArrayCGH). Unfortunately, we did not manage to find any candidate gene that could explain the OAT (not published).
In the course of these investigations we encountered several pitfalls in studying male infertility. First, not the whole process of spermatogenesis has been yet elucidated. Second, male infertility is heterogeneous in phenotype and (genetic) cause. Therefore, it was decided to focus my thesis research on morphological uniformity of the sperm cells of subfertile men in order to increase the chance of identifying a common genetic aetiology.

BOX 1 A family with globozoospermia.

In the search for families with several members suffering from the same type of male subfertility we identified a consanguineous family (figure 1) in which several male members appeared to suffer from globozoospermia (round headed sperm cells).

The proband suffered from severe OAT (concentration (C): 2.6-11 * 10^6 sperm cells/ml; motility (M): 10-30%: normal morphology (Morph): 0% and an increased amount of acrosomeless sperm cells (88-96%: Figure 2). He and his wife managed to conceive after one ICSI cycle, upon which she delivered a healthy son.

The parents of the proband, as well as two maternal uncles, also underwent assisted reproductive techniques. The parents of the proband were first cousins, as were the maternal parents. In addition, there was a possible consanguineous loop via the paternal parents as well. Together, the pedigree structure is suggestive of an autosomal recessive gene defect underlying the OAT in this family, although autosomal dominant or X-linked inheritance cannot be excluded. The parents of the proband underwent in vitro fertilisation (IVF) because of a tubal obstruction (double tubectomy because of multiple ectopic pregnancies) in order to conceive the younger brother of the proband. The father had normal semen parameters (C: 135*10^6/ml; M: 80%; Morph: 42%). The first maternal uncle suffered from a fertility problem for 8 years. His first child was conceived by Intra uterine insemination (IUI), he had normal semen parameters (C: 64*10^6/ml: M: 30%, Morph: unknown). His other 3 children were conceived in a natural way. The second maternal uncle suffered from disturbed semen parameters as well (C: 11-60*10^6/ml; M: 20-60%; Morph: 1%) as well as 75% acrosomeless sperm cells. He and his wife underwent ICSI-treatments in order to conceive their first three children; the fourth and last child was conceived in a natural way. The third and youngest maternal uncle also suffered from this dysmorphic sperm syndrome (C: 35*10^6/ml; M: 30%; Morph: 18%; 26% acrosomeless sperm cells). He and his wife conceived in a natural way with a prolonged time to pregnancy of 18 months. Because of the consanguinity, we assumed that this sperm morphology disorder was caused by an autosomal recessive condition. The family was therefore subjected to linkage analysis, which unfortunately did not reveal any common homozygous regions shared by the affected males, which could help to locate candidate genes. As time progressed, a 10 K SNP array was used for homozygosity mapping, but also this did not reveal homozygous candidate regions in the family. Our last option was to involve the younger brother of the proband in the study, who reached maturity during the time of research, but he was unwilling to cooperate. At that point we decided to cease our research in this specific family.

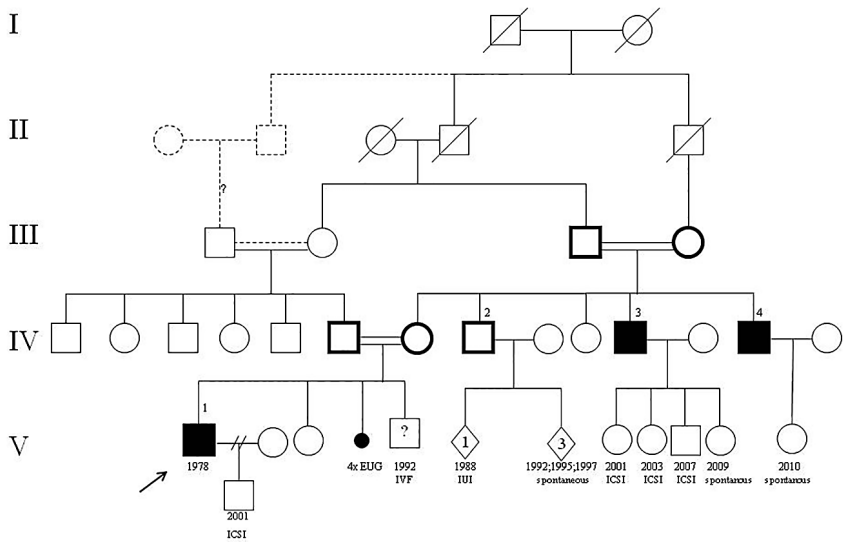

Figure 1 Family tree of the family with partial globozoospermia described in Box 1.

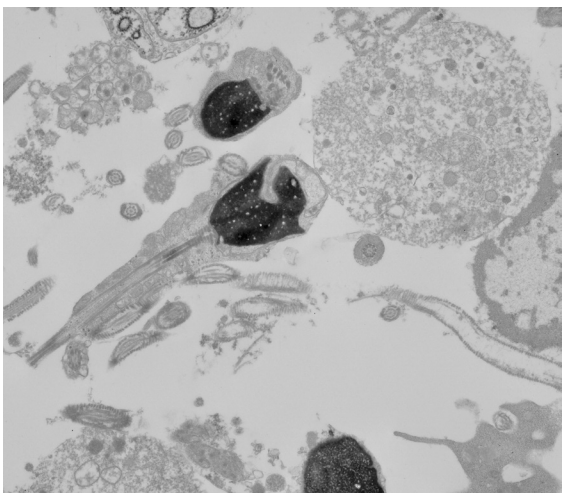

Figure 2 Partial globozoospermia, TEM image.

In this image an example of a malformed acrosome with invagination is shown as example of the acrosomic malformations that occur in partial globozoospermia.

PARTIAL GLOBOZOOSPERMIA

The story in Box 1 is typical for genetic research in male infertility: major efforts with minor results. The observations in the specific morphological syndrome in these patients led however to further studies. These patients namely did not suffer from total globozoospermia (100% acrosomeless) as described by Schirren *et al.* [15] but had a mild form of globozoospermia (called partial globozoospermia) in which only a variable part of the spermatozoa was round-headed and acrosomeless (Figure 2). Although this particular morphology has already been mentioned in literature[16], it has never been described properly. In our experience, *partial* globozoospermia occurs more often than total globozoospermia, which is a rare condition that occurs in less than < 0.1 % in cases of male infertility. I therefore decided to continue my research on partial globozoospermia.

BOX 2 Spermatogenesis, spermiogenesis and acrosome function.

Spermatogenesis (Figure 3) is the whole process that transforms spermatogonial stem cells into a spermatozoon. The whole process takes 74 days. It takes place within the germinal epithelium of the seminiferous tubules where germ cells are arranged along the wall of tubules in a well-defined combination of various developmental stages (Figure 4). At first, the mitotic phase takes place at the basal membrane, where the Type A spermatogonia on the base membrane divide asymmetrically into another Type A spermatogonia and Type B spermatogonia. The type B spermatogonia go into a phase of proliferation and transform into primary spermatocytes, which go into meiosis I. The resulting diploid secondary spermatocytes go into meiose II to become haploid round spermatids. Finally, there is a gradual remodeling of the chromatin and cellular components of round spermatids during transformation into sperm cells by a process referred to as spermiogenesis [17].

Spermiogenesis is thus the process by which the final sperm shape is reached. This last phase of spermatogenesis has been described by Clermont et al (1963) as a complex process that is divided in six steps (Sa, Sb-1, Sb-2, Sc, Sd-1, Sd-2) and four phases (table 1). [17, 18].

In the *Golgi-phase* (Sa), acrosome formation starts with the formation of acrosomic vesicles that are produced by the Golgi apparatus (Figure 4). These vesicles land on and align with the acroplaxome, an actin-keratin- containing cytoskeletal plate that acts as a scaffold that anchors the developing acrosome to the nuclear envelope [19].

BOX 2 Continued.

In the *Cap-phase* (Sb1-2) the fusing of the acrosomic vesicles takes place. A perinuclear ring is formed in which a manchette of microtubules is anchored to provide transport of golgi-derived acrosomal and non-acrosomal vesicles that play a role in sperm head elongation. On the contralateral side the centrioles migrate to form the axoneme, from which the tail is formed. Subsequently, the acrosomic granule enlarges, starts to flatten and is separated from the Golgi apparatus. The process of chromatin condensation is observed from step Sa onwards. This specific process serves the purpose to protect the paternal genome during transport through the female genital tract. The process consists of several steps in which somatic histones are replaced by sperm histones, to be replaced by transitions proteins 1 and 2 (TP) and finally by protamines 1 and 2 for 85% [20]. Chromatin condensation starts at the apical (acrosomal) pole of the spermatid nucleus by acetylation and deacetylation of histone H4, which is needed to start protamination [21, 22]. Transition protein 2 is already observed from step Sa [20]. Transient DNA strand breaks occurs to allow the replacement of histones and transition proteins by protamines, a phenomenon which has been observed using TUNEL in step Sb2, but not in the following steps, suggesting this phenomenon to be a part of chromatin condensation [23].

During the *Acrosomal phase* (Sc) the acrosome becomes hemispherical and chromatin condensation continues. Protamines further condense the DNA strand and form the donut-shaped packaging units called toroids. In human, 5-15% of the histones are retained. Protamines (P1 and P2) are normally expressed equally (1:1) in fertile sperm [24]. These sperm-specific proteins have an arginine core, that cause a positive charge that facilitates a strong DNA binding, and cysteine residues, that facilitate disulfide bonds within and in between the protamines, thus creating a condensed chromatin packaging. Protamine 2 contains less cysteine and therefore a low P1/P2 ratio is associated with infertility [24]. The retained histones are not randomly distributed and may have an important role during fertilization and the first cell divisions [25].

In the final *Maturation phase* (Sd1-2) the acrosome condenses, the mitochondria are arranged around the midpiece to provide the energy needed for the tail. The remaining cell organelles are discarded with the cytoplasmic body. H4 acelylation stops at Sd-1, as proven by in situ hybridisation [21]. The sperm is released into the lumen of the seminiferous tubule and leads to the epididymis for the process of epididymal maturation to become a motile spermatozoon [26].

BOX 2 Continued.

The *acrosome* is built in about 14 days and plays an important role in sperm-egg binding during the fertilisation process [17]. It is considered a secretory granule that consists of a acrosomal matrix that contains proacrosin as well as the acrosomal content that contains acrosin and other enzymes. When released at ejaculation into the female genital tract, the spermatozoon is subjected to *capacitation*, a process in which, amongst others, the proteins and glycoproteins of the seminal fluid are removed, and the sperm surface molecules are reorganized in order to induce an increased fluidity in permeability of the sperm plasma membrane. This process, which is not yet completely understood, prepares the spermatozoon to be responsive for the *acrosome reaction*. This calcium dependent induced reaction is initiated in (the proximity) of the cumulus cells. On binding to the zona pellucida, the sperm undergoes acrosomal reaction. The acrosome content is released in order to be able to penetrate the zona pellucida of the oocyte and expose the inner acrosomal membrane to fuse with the oolema. Needless to say that the acrosome plays a crucial role in natural fertilisation, without this step, no sperm-oocyte fusion can be achieved [17].

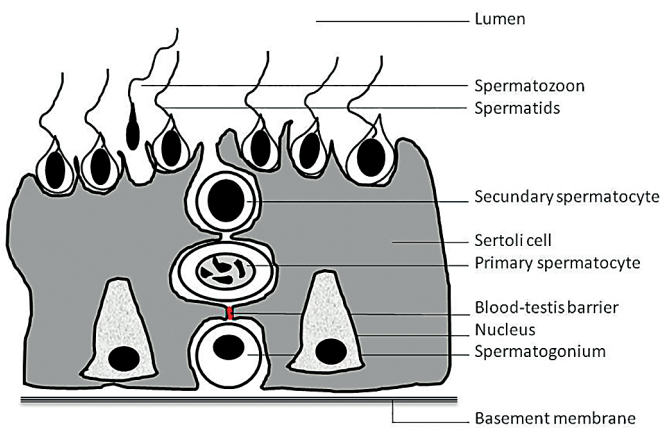

Figure 3 Spermatogenesis.

In this image a crossection of a part of the seminiferous tubule is pictured. The developement from the Type B spermatogonium to the mature spermatozoon in between the Sertolicells is schematically displayed.

Phase	Step	Events
Golgi-phase	Sa	• Formation, accumulation and fusion of proacrosomic granules from Golgi apparatus. • Association acrosomic vesicle to nucleus
Cap phase	Sb1-2	• Enlargement of acrosomic granule by glycoproteins from the Golgi apparatus. • Acrosome grows and flattens, head cap is formed. • Separation from the Golgi apparatus.
Acrosome phase	Sc	• Application to inner acrosomal membrane, the acrosome becomes hemispherical • Nucleus elongates, condenses and shifts towards the cell surface. • Formation cytoplasmic lobe
Maturation phase	Sd1-2	• Condensation of the acrosome • Mitochondria bind to middle piece of the tail • Discarding cytoplasmic droplet

Table 1 Spermiogenesis.

In this table the different phases, the coherent steps and the most important events during the phases are displayed.

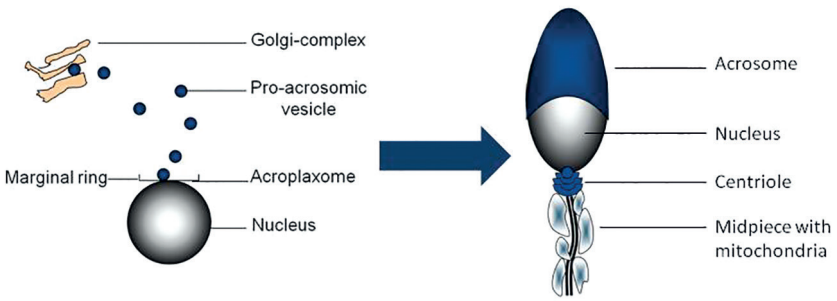

Figure 4 Schematised depiction of Golgiphase and mature spermatozoon.

Proacrosomic vesciles are produced by the Golgiapparatus, to land on the acroplaxome. There the vesicles fuse to form the acrosome that stretches over the elongating nucleus. In the end an oval shaped spermatozoon with an organized midpiece with mitochondriae is developed.

AIM OF THE STUDY

In our search for a uniform phenotype to identify genetic factors underlying male subfertility, we encountered a new sperm morphology disorder. We named it 'partial 'globozoospermia. After completing the study of the family described in Box 1, we identified more patients with apparently the same sperm morphological characteristics. The aim of the work described in this thesis is to identify the genetic defects underlying this specific morphological abnormality of sperm cells associated with subfertility and to characterize and describe (partial) globozoospermia in a cellular, molecular, and clinical way.

OUTLINE OF THE THESIS

Chapter 1 Gives a broad introduction including a description of a consanguineous family with partial globozoospermia.

→Part I: Globozoospermia
Chapter 2 In this chapter I collected all available literature on globozoospermia in a review article to provide a base for the further research. In this chapter all reported morphology characteristics, epidemiology, clinical features, etiology, sperm integrity, and genetic backgrounds are described.

→Part 2: Partial globozoospermia
Chapter 3 Describes the morphology studies of partial globozoospermia in 10 patients in comparison to three total globozoospermic patients and nine normozoospermic controls. We performed this study in order to determine whether partial globozospermia is a seperate kind of teratozoospermia next to total globozoospermia.

Chapter 4 Describes the study on sperm DNA integrity in which partial globozoospermia was compared to nine normozoospermic controls. This study was performed in order to evaluate the status of DNA damage in partial globozoospermia.

Chapter 5 Describes the reproductive outcome in 42 couples with male partners with partial globozoospermia (spontaneous pregnancy/IUI/IVF/ICSI). In this study the clinical performance in partial globozoospermia was established based on a case control study on ICSI of 27 partial globozoospermia cases in comparison to 263 ICSI controls.

→Part 3: Genetics
Chapter 6 Describes the search for a responsible gene and the mutation in *SPATA16* in a family suffering from total globozoospermia.

General discussion, Summary and appendices
Chapter 7 In the general discussion sperm morphology disorders, partial globozoospermia and the current status and future of genetic research in spermatogenesis are discussed.

Chapter 8 Summarizes chapter 2 to 7 in English.

Chapter 9 contains the Dutch summary; the NCEBP list of theses since 2000, the acknowlegements and Curriculum vitae

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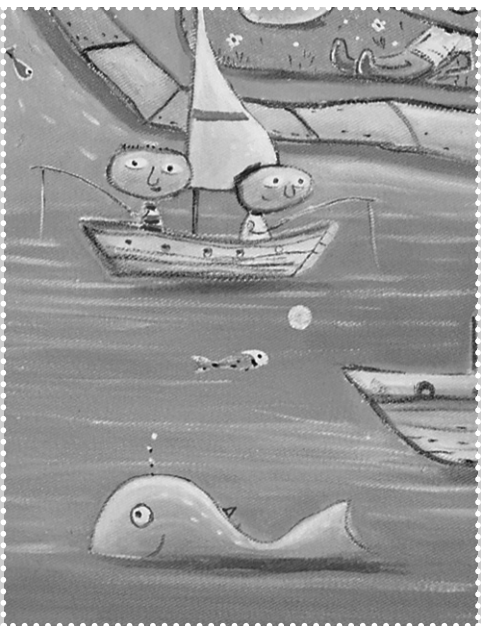
Part 1

Globozoospermia

Chapter 2

Globozoospermia revisited



Dam A.H.D.M., Feenstra I., Westphal J.R., Ramos L.,
van Golde R.J.T., Kremer J.A.M.

Hum Reprod Update, 2007. Jan-Feb;13(1):63-75.

ABSTRACT

Globozoospermia is a rare (incidence <0.1%) but severe disorder in male infertility. Total globozoospermia is diagnosed by the presence of 100% round-headed spermatozoa lacking an acrosome. It is still unclear whether patients whose ejaculate contains both normal and globozoospermic cells (partial globozoospermia) suffer from a variation of the same syndrome. Apart from the fact that affected males suffer from reduced fertility or even infertility, no other physical characteristics can be associated with the syndrome. ICSI is a treatment option for these patients, although low fertilization rates after ICSI show a reduced ability to activate the oocyte. In globozoospermic cells, the use of acrosome markers has demonstrated an absent or severely malformed acrosome. Chromatin compaction appears to be disturbed but is not consistently over- or undercondensed. In some cases, an increased number of cells with DNA fragmentation have been observed. The analysis of the cytogenetic composition revealed an increased aneuploidy rate in some cases. Nonetheless, no increased number of spontaneous abortions or congenital defects has been reported in pregnancies conceived after ICSI. The pathogenesis of globozoospermia most probably originates in spermiogenesis, more specifically in acrosome formation and sperm head elongation. In several knockout mouse models, a phenotype similar to that in humans was found. Together with the occurrence of affected siblings, these findings indicate a genetic origin, which makes globozoospermia a good candidate for genetic analysis. More research is needed to elucidate the pathogenesis of human globozoospermia to further understand globozoospermia as well as (abnormalities in) spermiogenesis and spermatogenesis in general.

Key words: acrosome/genetics/globozoospermia/male infertility/round-headed sperm cells

INTRODUCTION

Globozoospermia is a rare but severe disorder causing male infertility. Schirren et al. (1971) were the first to properly describe this syndrome of round-headed spermatozoa that lacked an acrosome. In the following three decades, a limited number of additional case reports were published. In these case reports, the morphological and aetiological aspects of globozoospermia were emphasized. However, the underlying causes of the syndrome still remain to be elucidated. A genetic contribution was postulated by Kullander and Rausing, which was supported by additional case reports of affected brothers (Kullander and Rausing, 1975; Florke-Gerloff et al., 1984; Dale et al., 1994; Carrell et al., 1999, 2001; Kilani et al.,2004). The gene(s) responsible or the mode(s) of inheritance remains obscure.

It is clear that male infertility in general is based on a multifactorial aetiology and tends to cluster in families. Diverse inheritance patterns have been described (Gianotten et al., 2004; van Golde et al., 2004). Genetic analysis has been complicated by the lack of a distinct correlation between genotype and (a highly variable) phenotype. The first description of globozoospermia, presenting as a disorder that affected all spermatozoa of a patient in a very specific way (Schirren et al., 1971), suggested that globozoospermia was an exception. The apparently very distinct phenotype suggested a monogenetic trait, thereby rendering this syndrome into an attractive target for genetic studies. Subsequent reports, however, presented a more diverse phenotype, obscuring the initial clear-cut phenotype (Holstein et al., 1973; Anton- Lamprecht et al., 1976; Florke-Gerloff et al., 1984).

Morphological defects in human sperm cells raise suspicion for further anomalies within the sperm cell and could have implications in clinical practice. Therefore, in this review we have attempted to summarize the phenotypic manifestations, and possible causes of the condition as well as implications for clinical practice of globozoospermia, as a prerequisite for genetic analysis.

MATERIALS AND METHODS

For this systematic review, we collected all papers published on globozoospermia from 1965 to January 2006, using the following keywords [globozoosperm* OR (round AND headed AND sperma*) OR (round AND headed AND sperm) OR acrosomeless].

The initial literature search was performed using PubMed, Scirus and Medline. English was used as a limit, except for the Scirus database, because language

selection is not possible in this database. Regular updates were performed using the same keywords in PubMed up until January 2006. The initial search resulted in 88 hits in PubMed, 72 hits in Medline and 148 journal hits in Scirus. Subsequent searches in PubMed revealed another 11 papers. Papers dealing with megalohead sperm cells, round spermatids, macronuclear spermatozoa or acrosome malformation were excluded. In addition, we excluded papers in which cases of globozoospermia were used or proposed for research purposes, without specifically focusing on globozoospermia, nor providing additional information on globozoospermia (Kaufmann et al., 1987; von Bulow et al., 1995; Dimitrova-Dikanarova et al., 1998; Lefievre et al., 2003). Scirus appeared to be less accurate and produced a lot of hits that did not deal with globozoospermia or formed duplicates; these papers were excluded as well. After this selection, 80 PubMed, 60 Medline and 69 Scirus papers remained. The hits generated by PubMed included the 60 Medline papers. Scirus found seven papers that were not found by PubMed, resulting in 87 papers selected for review. From the reference list of the selected papers, four German papers were selected to be included in our review, because they either mentioned the occurrence (Meyhöfer, 1965), features and pathogenesis of round-headed sperm cells (Schirren et al., 1971; Holstein et al., 1973) or suggested the term globozoospermia for the first time (Wolff et al., 1976). For background information see the Handbook of Andrology (http://www.andrologysociety.com/ resources/handbook.aspx).

RESULTS

Morphological description

The first to mention 'Rundkopfspermatozoen' (round-headed spermatozoa in German) after light microscopic analysis was Meyhöfer (1965). In 1971, Schirren *et al*. described the fine structure of these round-headed sperm cells as determined by electron microscopy (Figure 1) and discovered that their round shape was caused by a round nucleus lacking an acrosome. Other groups reported similar findings (Pedersen and Rebbe, 1974; Kullander and Rausing, 1975; Wolff *et al*., 1976; Baccetti *et al*., 1977, 1981), of whom Wolff *et al*. first suggested the term 'globozoospermia'. Table I summarizes the reported morphological characteristics of globozoospermia.

Globozoospermia is normally diagnosed by the detection of round-headed sperm heads during routine light microscopic examination of a semen sample. The absence of an acrosome is another major feature, which is best visualized by transmission electron microscopy. Furthermore, sperm cells tend to have multiple defects involving the cytoskeleton such as a round nucleus, absence of

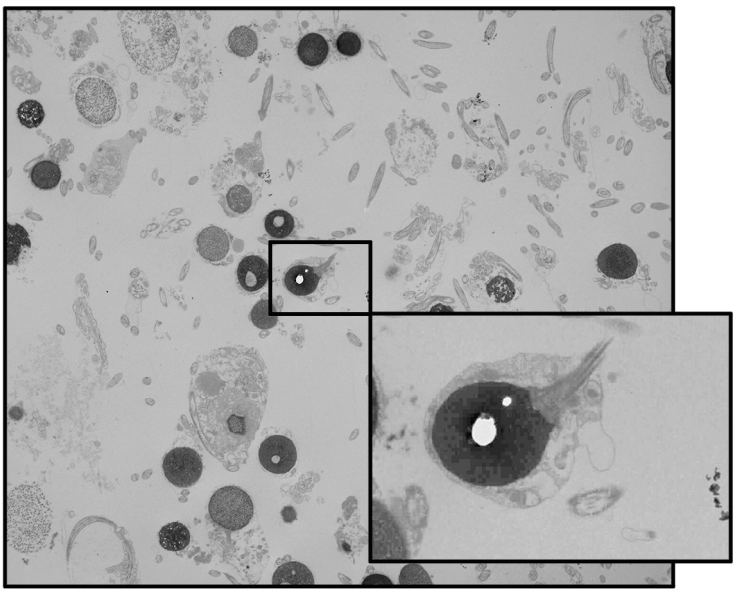


Figure 1 Globozoospermia.

Overview imaged by Transmission Electron microscopy (TEM).
In detail a globozoospermic sperm cell is shown. This cell clearly shows the main characteristics of globozoospermia, such as round headed nucleus and the lack of an acrosome. Also the cytoplasmic droplet is evident.

the post-acrosomal sheath, separation of the nuclear membranes and frequently coiled tails. Also maturation defects such as a persisting residual cytoplasmic body or droplet surrounding the nucleus or the midpiece have frequently been reported.

Most of the papers based on describing this morphology deal with patients who produced ejaculates showing 100% round sperm heads lacking an acrosome. Holstein et al. (1973), however, reported a number of patients who only had 20–60% round-headed spermatozoa in their ejaculate. Whether this 'partial' globozoospermia was a different aspect of the same syndrome or a separate disorder could not be determined. The matter was further complicated by a case report by Anton-Lamprecht et al. (1976), who reported two types of patients with round-headed sperm cells. The first patient was of the classic 'Schirren–Holstein type', with exclusively round-headed acrosomeless sperm cells as determined by light and electron microscopy. The ejaculate of the second patient showed 80% of round-headed sperm cells, as determined by light microscopy, but

100% round headed (50)	Disturbed midpiece (4)
Acrosomeless (42)	Abnormally arranged mitochondria (4)
<100% round headed (19)	Acrosome separated from nucleus (2)
Round nucleus (14)	Post-nuclear ring absent (2)
No postacrosomal sheath (12)	Chromatin treads (2)
Coiled tails (partially) (11)	Atypical implantation of the sperm tail (1)
Abnormally condensed nucleus (9)	Double heads (1)
Vacuoles in nucleus (6)	Tail abnormalities (1)
Separated nuclear envelope (6)	Invagination (1)
Cytoplasmic droplet (6)	

Table I Overview of reported morphological characteristics of globozoospermia.

The order is established by the frequency of description; the quantity of references in which the characteristic was mentioned is indicated as acrosomeless (42).

electron microscopy showed that the round-headed phenotype was due to a residual cytoplasmic body that surrounded the nucleus and acrosome. They suggested the terms 'globozoospermia type I' for the classic Schirren–Holstein phenotype, and 'globozoospermia type II' for the phenotype observed in the second patient. Although their first suggestion was adopted in literature, only two globozoospermia type II patients have been reported since (Christensen et al., 2006). However, patients with <100% round-headed sperm cells in their ejaculate have frequently been described (Pedersen and Rebbe, 1974; Weissenberg et al., 1983; Florke-Gerloff et al., 1984; Syms et al., 1984; Tyler et al., 1985; Singer et al., 1986; Lanzendorf et al., 1988; Rybouchkin et al., 1996, 1997; Carrell et al., 1999, 2001; Coetzee et al., 2001; Larson et al., 2001).

To complicate matters even further, a number of cases were described in which electron microscopic examination showed that the sperm cells in these patients that did not appear to be round- headed by light microscopy could nonetheless lack an acrosome (Pedersen and Rebbe, 1974; Tyler et al., 1985; Lanzendorf et al., 1988; Coetzee et al., 2001). In other cases, only the round-headed sperm cells were found to be acrosomeless (Syms et al., 1984; Singer et al., 1986; Carrell et al., 1999). This was confirmed by immunofluorescent techniques using antibodies against (pro-) acrosin (Florke-Gerloff et al., 1984). Even if sperm cells were of normal or otherwise abnormal shape, an acrosome often appeared to be missing or anomalous (Florke-Gerloff et al., 1984; Rybouchkin et al., 1996; Carrell et al., 2001; Larson et al., 2001). According to these findings, it is clear that these cases do not belong to type II as described by Anton-Lamprecht et al. (1976).

Hence, this nomenclature could be misleading. We therefore suggest the term 'partial globozoospermia' for patients in which <100% of the sperm cells show a round-headed form without an acrosome. Whether classic/total globozoospermia and partial globozoospermia are part of the same syndrome remains to be elucidated, today as urgently as >30 years ago.

Epidemiology

The first case of globozoospermia, described by Schirren et al. (1971), was found after the examination of 2200 patients undergoing routine andrological screening, indicating an incidence of <0.05%. Later, the same group corrected the incidence to 0.1% among andrological patients (Holstein et al., 1973). Subsequent reports adopted this estimated incidence (Schill, 1991; Coetzee et al., 2001; Kim et al., 2001; Kalahanis et al., 2002). The documentation of globozoospermia, however, did not go beyond the level of case reports, so this incidence might be overestimated.

Round-headed cells also occur in otherwise normal semen samples (Florke-Gerloff et al., 1984). In the ejaculate of a proven fertile man, Florke-Gerloff et al. (1984) observed up to 6% of round-headed sperm cells. These cells lacked acrosin and the outer acrosomal membrane. Andrade-Rocha examined 233 suspected infertile men. The percentage of round-headed sperm cells in their ejaculates varied from 0.1 to 0.8%. This percentage was, in contrast to the percentage of abnormal sperm cells in general, not significantly related to the sperm count (Andrade-Rocha, 2001). Finally, Kalahanis et al. (2002) examined semen from 114 subfertile (≥2 years unvoluntary childlessness without a female cause) and 60 proven fertile men. The percentage of round-headed sperm cells was slightly, but significantly higher in subfertile men (2.3 ± 0.5%) compared to fertile men (0.5 ± 0.1%) (Kalahanis et al., 2002). In relation to environmental factors, Rubes et al. (1998) concluded that smokers show a significantly increased amount of round-headed sperm cells in their ejaculate, in comparison with non-smokers. Although this was a relatively small study (20 smokers versus 15 non-smokers), these results might form an indication for an environmental factor in partial globozoospermia.

Clinical parameters

According to our search, 99 cases of globozoospermia have been reported since the first Schirren case. In this review, we summarize the clinical parameters of the patients involved. The mean age of the patients, as reported in 70 of 99 cases, was 34.7 years, and 68% of the men were between 30 and 40 years old when examined. Semen parameters were described in detail in 72 cases and showed great variation. Figure 2 shows the reported values in volume, concentration

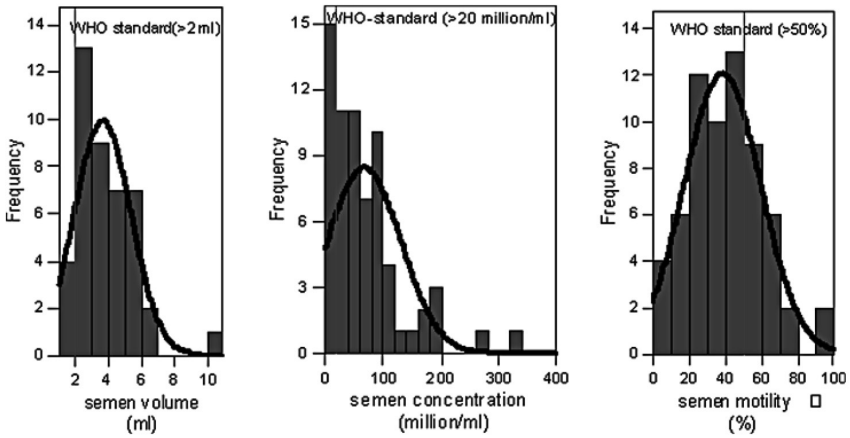


Figure 2 Semen parameters in 72 men with globozoospermia.

Semen volume (n = 43) mean = 3.7 ml. Sperm concentration (n = 67) mean = 67 × 106/ml. Sperm motility (n = 64) mean = 39%.

and motility, respectively. Besides a general observation of slight asthenozoospermia and the typical morphological characteristics, no abnormalities in semen parameters could be characterized as a typical feature of globozoospermia. Closer investigations by a computerized digital image analysis system revealed no abnormalities in sperm movement characteristics in globozoospermia (Aitken et al., 1990).

In 16 of 99 cases, a somatic karyotype of the affected patient was determined. A normal male somatic karyotype was found in 15 cases, one case displayed a mosaic trisomy 21 in 17.5% of the somatic cells (Kim et al., 2001). On the level of DNA, a microdeletion of the Y-chromosome was found in another patient (Zeyneloglu et al., 2002). Gunalp et al. (2001), however, did not find any Y-chromosome microdeletions in 12 cases of globozoospermia.

In summary, these data indicate that patients with globozoospermia in general carry a normal karyotype. The incidence of Y-chromosome microdeletions in globozoospermia does not appear to exceed the incidence in male subfertility in general.

The results of andrological history or examination were reported in 31 cases. In 22 cases, a normal health, normal masculine development and/or a normal aspect of the genitalia were reported (Pedersen and Rebbe, 1974; Kullander and Rausing, 1975; Anton-Lamprecht et al., 1976; Weissenberg et al., 1983; Jeyendran et al., 1985; Santi de et al., 1985; Lalonde et al., 1988; von Bernhardi et al., 1990;

Dale et al., 1994; Bourne et al., 1995; Kilani et al., 1998, 2004; Viville et al., 2000; Nardo et al., 2002; Vicari et al., 2002; Pirrello et al., 2005). The remaining nine cases included four (treated) varicoceles (Florke-Gerloff et al., 1985; Jeyendran et al., 1985; Pirrello et al., 2005), two orchidectomies (Jeyendran et al., 1985; Pirrello et al., 2005), two (gonorrhoeal) infectious epidydimitis (Kullander and Rausing, 1975; Singer et al., 1986) and one unspecified penile ulcer in the past (Pedersen and Rebbe, 1974).

Aberrant details were sporadic, including a globozoospermic patient with bronchiectasis (Santi de et al., 1985), two cases of toxic exposition (Jeyendran et al., 1985; Carrell et al., 1999), two cases of consanguinity (Kilani et al., 2004; Pirrello et al., 2005) and two cases of opiate addiction in the past (Florke-Gerloff et al., 1985; Singer et al., 1986). Parotitis was mentioned in three cases, although orchitis did not occur in any case (Schirren et al., 1971; Kullander and Rausing, 1975), thereby excluding parotitis as a causing factor. The inconsistent occurrence of these physical characteristics leads to the conclusion that next to reduced fertility, there are no other characteristics correlated with the globozoospermia phenotype.

(In)Fertility

In 64 cases, the patient was reported to be infertile. When Schirren et al. (1971) discovered that in case of globozoospermia all spermatozoa were acrosomeless, this was considered to be the cause of the infertility. Remarkably, proven fertility was noted in two separate cases. In the first case, a pregnancy was reported from a couple of whom the male partner presented with 'type II' globozoospermia (Anton-Lamprecht et al., 1976). The second case suffered from a 3-year involuntary childlessness, but this episode was preceded by two subsequent spontaneous abortions. No cause, explanation or evidence for acquired total globozoospermia was formulated (Arrighi et al., 1980). It should be noted that in neither case a paternity test was performed. Therefore, the involvement of a third party cannot be excluded.

The introduction of ICSI (Palermo et al., 1992) provided a solution in fertility treatment for patients suffering from globozoospermia (Hamberger et al., 1998). In 1994, Lundin et al. reported the first pregnancy and delivery using globozoospermic, acrosomeless spermatozoa in a second ICSI cycle. Since then, numerous reports have described successful attempts to achieve either fertilization or pregnancy following ICSI with globozoospermic sperm cells (Bourne et al., 1995a,b; Liu et al., 1995; Trokoudes et al., 1995; Battaglia et al., 1997; Rybouchkin et al., 1997; Kilani et al., 1998, 2004; Stone et al., 2000; Coetzee et al., 2001; Kim et al., 2001; Nardo et al., 2002; Tesarik et al., 2002; Zeyneloglu et al., 2002; Heindryckx et al., 2005). All reports are summarized in Table II. In general, ICSI with globozoo-

Reference	Patient	Treatment	Fertilization		ET	Outcome
			Abs	%		
Lundin *et al.* (1994)	One couple, 100% globozoospermia	Two cycles ICSI	13/5	38	2 ET	–
			15/7	47	2 ET	+ twins
Bourne *et al.* (1995a,b)	One couple, 100% globozoospermia	One cycle ICSI	10/3	38	2 ET	–
Liu *et al.* (1995)	Seven couples, 100% globozoospermia	ICSI	1.5/0	0	No ET	
		ICSI	1.1/0	0	No ET	
		ICSI	1.2/0	0	No ET	
		ICSI	2.19/5	26	3 ET	–
		ICSI	3.25/1	4	1 ET	+ abortion
		ICSI	4.1/0	0	No ET	
		ICSI	4.3/0	0	No ET	
		ICSI	5.11/3	27	3 ET	+ EUG
		ICSI	5.7/5	71	3 ET	+ twins
		ICSI	6.5/0	0	No ET	
		ICSI	7.6/0	0	No ET	
Trokoudes *et al.* (1995)	One couple, 100% globozoospermia	IVF	0			
		SUZI	0			
		ICSI	6/3	50	2 ET	+ singleton
Battaglia *et al.* (1997)	One couple, 100% globozoospermia	ICSI	15/1	7		
		+ OA 18 h pi	14/11	79	5 ET	–
		ICSI	20/2	10		
		+ OA 30 min pi	8/6	75		
		+ OA 20 h pi	15/11	73	5 ET	+ abortion
Rybouchkin *et al.* (1997)	One couple, 90% type 1 globozoospermia	ICSI	15/1	7	No ET	
		ICSI + OA	5/5	100	3 ET	+ singleton

Kilani *et al.* (1998)	One couple, 100% globozoospermia	ICSI	7/3	43	3 ET	–
		ICSI	4/2	50	2 ET	–
		ICSI	9/8	89	4ET	+ triplet
Stone *et al.* (2000)	One couple, 100% globozoospermia	ICSI	10/4	40	3 ET	–
	Endometriosis					
		ICSI	11/1	9	1 ET	–
		ICSI	19/8	42	3 ET	+ singleton
Coetzee *et al.* (2001)	One couple, 99% globozoospermia	ICSI	7/3	43	3 ET	+ twins
Kim *et al.* (2001)	One couple, 100% globozoospermia mosaic	ICSI + OA + assisted hatching	35/21	60	5 ET	–
	Down					
		Frozen embryos			5 ET	+ singleton
Nardo *et al.* (2002)	Two couples, globozoospermia	ICSI	1.7/3	43	3 ET	+ singleton
		ICSI	2. 5/2	40	2 ET	–
Tesarik *et al.* (2002)	Two couples, 100% globozoospermia	ICSI + OA	1.8/6	75	2 ET	–
		ICSI modified	1.11/9	82	3 ET	+ singleton
		ICSI + OA	2.4/4	100	No ET	
		ICSI modified	2.3/2	67	2 ET	–
Zeyneloglu *et al.* (2002)	One couple, 100% globozoospermia Y	ICSI	13/4	31	4 ET	+ twins

Table II Fertility treatment, fertilization and pregnancy rates in globozoospermic patients.

Abs, fertilization in absolute figures; AOA, artificial oocyte activation; ET, embryo transfer/number of embryos' transferred; outcome; pregnancy +/–; OA, oocyte activation; SUZI, subzonal insemination; pi, post insemination.

Reference	Patient	Treatment	Fertilization		ET	Outcome
			Abs	%		
	Microdeletion					
Kilani *et al.* (2004)	Five brothers, 100% globozoospermia	ICSI	1.3/1	33	1 ET	–
		ICSI	1.8/0	0	No ET	
		ICSI	1.12/4	30	4 ET	–
		ICSI	1.10/4	40	4 ET	–
		ICSI	1.8/2	20	2 ET	–
		ICSI	1.6/3	50	3 ET	–
		ICSI	2.8/6	75	4 ET	–
		ICSI	2.7/2	28	2 ET	–
		ICSI	2.10/3	30	2 ET	–
		ICSI	3.2/2	100	2 ET	–
		ICSI	3.3/1	33	1 ET	–
		ICSI	3.1/0	0	No ET	
		ICSI	3.1/1	100	1 ET	–
		ICSI	4.4/2	50	2 ET	–
		ICSI	4.10/3	30	3 ET	–
		ICSI	4.12/1	8	1 ET	+ abortion
		ICSI	5.9/6	66	5 ET	+ singleton
		ICSI	5.2/2	100	2 ET	–
		ICSI	5.6/4	66	3 ET	+ abortion
		ICSI	5.7/2	28	2 ET	–
Heindryckx *et al.*(2005)	Six couples, 100% globozoospermia	12 cycles ICSI + AOA	167/128	77	?	+/+
						+ singleton/ + twins
						+ singleton
						+ singleton
						+ singleton (cryo)

Table II Continued.

spermic cells is less successful compared with ICSI in general. Several authors reported a low to absent fertilization (Liu et al., 1995; Battaglia et al., 1997; Rybouchkin et al., 1997; Stone et al., 2000; Kilani et al., 2004). Rybouchkin et al. (1996) discovered that fertilization was improved by the addition of a calcium ionophore subsequently confirmed by several studies (Rybouchkin et al.,1996; Gomez et al., 2000; Schmiady et al., 2005).

As for the pregnancy outcome, Nagy et al. (1998) reported that although the fertilization rate was decreased in cases in which morphologically abnormal spermatozoa were used, no increase in the number of spontaneous abortions or congenital defects occurred. In accordance with these findings, no increase in such outcome failures has been reported in globozoospermia. Nevertheless, abnormal sperm morphology might be associated with genetic alterations in the sperm cells, which theoretically could have consequences on the long term for the offspring from artificial reproduction techniques. Sperm constitution in case of globozoospermia will be evaluated in the chapter 'molecular description'.

Pathogenesis

In globozoospermia, one or more of the sperm-remodelling mechanisms in spermiogenesis appear to be impaired. Especially, acrosome formation and nucleus elongation were studied in detail in globozoospermia patients. Four possible mechanisms have been postulated to explain the absence of the acrosome.

First, the acrosome may develop separately from the nucleus to be lost in the Sertoli cell.

Electron microscopic studies by Schirren et al. (1971) showed that the acrosome is present in early spermatids but develops in the cytoplasm, independently from the nucleus. In addition, the nucleus remained round, but no condensation abnormalities were reported (Schirren et al., 1971). Several authors confirmed these findings (Baccetti et al., 1977; Castellani et al., 1978; Nistal and Paniagua, 1978). The same group (Holstein et al., 1973) observed that the abnormally developed acrosome was removed with the residual body and left in the Sertoli cell, where it degenerated. This has been confirmed by the identification of remnants of abnormal acrosomal vesicles in the Sertoli cell cytoplasm (Castellani et al., 1978).

Second, the acrosomal vesicles do not fuse and even detach from the nuclear membrane. Results obtained with immunohistochemical techniques (Florke-Gerloff et al., 1985) are in accordance with the electron microscopic findings described above. These authors demonstrated the presence of three acrosomal markers (acrosin, intra-acrosin inhibitor and purified outer acrosomal membrane) in early globozoospermic spermatids. These markers were located adjacent to, but separated from, the nuclear membrane. Instead, they appeared to move into the cytoplasm and to be discarded together with the residual body.

Third, the acrosomal granules may be formed but degenerate subsequently. Baccetti et al. (1977) postulated this theory after the observation that the acrosomic vesicle does attach to the nuclear membrane but degenerates in the late spermatid stage, leaving a pouch of membranes and small vesicles located on top of the nucleus. Moreover, hypoplasia of the Golgi apparatus was found in most spermatids in case of globozoospermia. A malfunctioning Golgi apparatus was therefore postulated as a possible cause of this malformation of the acrosome (Baccetti et al., 1977; Castellani et al., 1978; Nistal and Paniagua, 1978).

Finally, the caudal manchette may be absent or malfunctioning (Baccetti et al., 1977; Castellani et al., 1978; Nistal and Paniagua, 1978). Naturally, a combination of these cytoskeleton impairments is possible as well (Lalonde et al., 1988; Escalier, 1990).

With regard to the round shape of the nucleus, Schirren et al. (1971) suggested that the nuclear shape was only determined by the acrosome, leaving the nucleus round-shaped in its absence. Subsequent studies, however, showed that the caudal manchette was often missing in developing spermatids of a globozoospermic male, indicating that the shape of the nucleus might be influenced by aplasia or hypoplasia of the caudal manchette as well (Baccetti et al., 1977; Castellani et al., 1978; Nistal and Paniagua, 1978). Longo et al. (1987) showed that calicin, a basic protein that is almost exclusively located to the posterior part or calyx of the sperm nuclear theca, appeared to be absent in globozoospermic cells. This finding indicates an impaired development of the sperm-specific skeleton (Escalier, 1990; Courtot, 1991), which may influence both the formation of the acrosome and the shape of the nucleus. Research in genetically altered mice has succeeded in elucidating some aspects of spermiogenesis in globozoospermia together with other kinds of teratozoospermia. This will be discussed in the last section of this article.

Functional aspects

Acrosomal markers

The apparent absence of the acrosome in globozoospermia initiated several studies to demonstrate a disturbance of the functional properties of the acrosome and its membranes. First, the location of acrosin, its precursor proacrosin and the outer acrosomal membrane at the acrosomal region was investigated in normal spermatozoa using an indirect immunofluorescent staining technique with antibodies specific to these acrosomal components. In normal spermatids, the acrosomal developmental pattern was in accordance with data obtained using the electron microscope. In contrast, both acrosin and the outer acrosomal membrane were absent in mature globozoospermic spermatozoa, and the developmental pattern of globozoospermic spermatids was disturbed compared

with normal spermatids. These findings were confirmed by testing acrosome function with the gelatinolysis test, which showed no proteolytic activity of the mature sperm-head of globozoospermic cells (Florke-Gerloff et al., 1983). Spectrophotometrically evaluated acrosin activity showed severely diminished to absent activity in globozoospermia (Florke-Gerloff et al., 1984, 1985; Lalonde et al., 1988). Jeyendran et al. (1985) found an eight times decreased amount of proacrosin, but the level of active acrosin after induced conversion appeared to be normal (Jeyendran et al., 1985). This suggests that the level of proacrosin was diminished, but yet capable of conversion.

Phospholipase A2, which is believed to play a role in the acrosome reaction by hydrolysing fatty acids, which are linked to membrane phospholipids overlying the acrosome. Its activity in globozoospermic cells was significantly lower compared with that of spermatozoa from donors of proven fertility (Lalonde et al.,1988).

Fluorescein-labelled lectins were used as acrosome-membrane markers in developing spermatids. The binding of peanut agglutinin (PNA), a marker for acrosome differentiation, to the developing acrosome was absent. Ricinus communis agglutinin II (RCA II), which displays a cap-like fluorescence pattern in normal spermatids, presented a dot-like pattern in globozoospermic spermatids. The authors concluded that the adhesion and penetration failure observed in globozoospermia might be caused by membrane defects (Wollina et al., 1989). In accordance with these findings, vesicle-associated membrane protein (VAMP) or synaptobrevin was only found in rudiment form on globozoospermic cells when used as an acrosome marker, again pointing at a defect acrosome formation (Ramalho-Santos et al., 2002).

In summary, these studies show a present, but disturbed acrosome genesis, resulting in either a severely malformed or an absent acrosome in globozoospermia.

Fertilization capacity

Literature indicates that no spontaneous fertilization occurs in case of total globozoospermia. Several authors tested the fertilization capacity of human round-headed spermatozoa in animal models. In summary, these spermatozoa were capable of penetrating the cervical mucus (Jeyendran et al., 1985) but did not succeed in fertilizing (zona-free) hamster oocytes, unlike spermatozoa of men of proven fertility (Weissenberg et al., 1983; Syms et al., 1984; Jeyendran et al., 1985; Sutherland et al., 1985; Lalonde et al., 1988; von Bernhardi et al., 1990). This was most probably because of the incapacity to bind to the zona pellucida, as well as to problems during the fusion process of the spermatozoon with the oocyte membrane. Globozoospermic spermatozoa also failed to adhere to human zona pellucida. As elevated intracellular Ca^{2+} levels were supposed to be

required for binding of the spermatozoon to the oocyte, binding capacity was also studied in the presence of the calcium ionophore A23187. Even then, however, the spermatozoa failed to adhere (Aitken et al., 1990; von Bernhardi et al., 1990; Carrell et al., 1999), which led to the conclusion that sperm fusion capacity cannot be triggered by increasing intracellular calcium (Dale et al., 1994).

Interestingly, the nuclei of round-headed spermatozoa do decondense to a similar degree as do normal spermatozoa when incubated with crushed hamster ova, indicating a normal fertilization capacity once the barrier of the oocyte membrane is overcome (Syms et al., 1984). Lanzendorf et al. (1988) elaborated on these findings by injecting human globozoospermic sperm cells into zona-intact hamster oocytes. They showed that the abnormal sperm was capable of apparently normal chromatin decondensation and pronucleus formation after injection (Lanzendorf et al., 1988). After the introduction of ICSI in humans (Palermo et al.,1992), fertilization and even pregnancy was achieved in several cases of globozoospermia (Lundin et al., 1994; Sathananthan,1994; Trokoudes et al., 1995). The success of ICSI as a treatment option for globozoospermia, however, was not universal, as successive case reports reported low to absent fertilization in cases of ICSI. A down-regulation of an oocyte-activating factor was suspected (Liu et al., 1995; Battaglia et al., 1997; Rybouchkin et al.,1997; Nagy et al., 1998; Gomez et al., 2000; Stone et al., 2000; Coetzee et al., 2001). Poor oocyte activation capability was confirmed by the observation of premature chromosome condensation of globozoospermic sperm cells (Edirisinghe et al., 1998; Schmiady et al., 2005). Interestingly, performing ICSI in the presence of the calcium ionophore A23187 appeared to be able to overcome the failure in oocyte activation (Liu et al., 1995; Battaglia et al.,
1997; Rybouchkin et al., 1997; Nagy et al., 1998; Gomez et al.,2000; Stone et al., 2000; Coetzee et al., 2001).

Several heterologous ICSI models have been developed to test the oocyte-activating capacity of spermatozoa after injection. In a mouse model, the human globozoospermic cells indeed failed to activate and fertilize the oocytes. When the oocytes were artificially activated with ethanol 8%, fertilization did increase to normal levels (Rybouchkin et al., 1996; Battaglia et al., 1997; Kim et al., 2001; Tesarik et al., 2002). A similar mouse oocyte activation test has been used in a clinical setting to predict the necessity of oocyte activation with a $CaCl_2/Ca^{2+}$ ionophore, and showed again poor fertilization of globozoospermic cells, overcome by the $CaCl_2/Ca^{2+}$ ionophore oocyte activation (Heindryckx et al., 2005).

Poor oocyte-activation capacity is thought to originate from the sperm centrosome as well, in its function as a microtubule-organizing centre. The function

of the centrosome was examined in bovine oocytes and showed low sperm aster formation rates in globozoospermia after ICSI, supposedly because of a dysfunctional centrosome. Surprisingly, ethanol-induced oocyte activation increased fertilization rates, suggesting a centrosome-independent sperm ability to activate the oocyte (Terada et al., 2001, 2004; Nakamura et al., 2002; Terada, 2004).

Viability

Sperm viability is normally tested by eosin staining, as recommended by the World Health Organization (WHO). Jeyendran et al. (1984), however, developed the hypo-osmotic swelling test, based on the observation that exposing living sperm with intact membranes to hypo-osmotic conditions (150 mOsm/l) results in fluid flux into the cell, thereby increasing the sperm volume and resulting in a visible swelling of the tail membrane. This hypo- osmotic swelling test (HST) was used in combination with eosin staining to test the viability of round-headed sperm cells. In two globozoospermia patients, no decreased percentage of viable sperm cells were encountered (Jeyendran et al., 1985; Check et al., 1993).

Molecular aspects

Morphological deformities are indicative of structural chromatin and DNA abnormalities and cytogenetic defects in spermatozoa. Chromatin condensation and stability, DNA fragmentation and aneuploidy rates have been investigated and evaluated in round-headed sperm cells, although it should be noted that in general the number of patients investigated in each study was low, resulting in rather anecdotal data sets.

Nuclear chromatin

Sperm chromatin is condensed during spermiogenesis, in which process, amongst others, histones are replaced by protamines (for a review, see Dadoune, 2003). Abnormal chromatin condensation is a strong indication of a maturation defect. The morphological aspects of chromatin condensation in globozoospermia have been described repeatedly in various (electron microscopic) studies. These reports, however, differ in their findings. Chromatin condensation was classified as normal, granular or fine treaded in early reports (Pedersen and Rebbe, 1974; Anton-Lamprecht et al.,1976; Castellani et al., 1978). Later descriptions report abnormally condensed chromatin, without further specification (Kullander and Rausing, 1975; Wolff et al., 1976; Tyler et al., 1985; Lalonde et al.,1988; Escalier, 1990; Singh, 1992; Battaglia et al., 1997; Carrell et al., 1999; Vicari et al., 2002). In addition, the evaluation of several factors involved in chromatin condensation was performed. Again, various results have been published. Arrighi et al. (1980) observed high heterogeneity in Feulgen DNA

contents within one patient, with low mean Feulgen stainability in globozoospermia, indicating overmaturity (Arrighi et al., 1980). Baccetti et al. (1977) based their conclusions on their previous findings that chromatin immaturity is characterized by high zinc, low phosphorus and variable lysine levels (Baccetti et al., 1977). Varying results were obtained within one patient. Although most cells showed immature chromatin by their definition, several cells were compact and mature as defined by low zinc and high phosphorus levels and by severely reduced lysine levels.

The presence of lysine was believed to indicate poor substitution of histones by protamines. Later reports indeed showed a disrupted replacement of histones by protamines (Blanchard et al., 1990). In one of two globozoospermia patients, a high histone rate in combination with a low protamine 1 (P1) percentage was found. The Protamine 2 (P2) percentage was low but not related to the histone content. In both patients, a decreased P1/P2 ratio (0.51 and 0.52, respectively in comparison with 0.59 in fertile controls) was found. By contrast, Contradictorily, an increased P1/P2 ratio (2.15) was found in one of two siblings suffering from globozoospermia (Carrell et al., 1999). In the other sibling, a normal ratio (0.79) in comparison with fertile controls was found. Chromatin structure measured by the flow cytometric sperm chromatin structure assay (SCSA), which is based on the susceptibility of sperm nuclear DNA to acid-induced denaturation, showed no differences in the chromatin stability of globozoospermic cells in comparison with highly fertile patients (Larson et al., 2001). Chromatin maturation can also be measured by propidium iodide staining, as immature chromatin is more accessible to this compound and is therefore more strongly stained. Vicari et al. (2002) reported an elevated number of globozoospermic sperm cells with immature chromatin in a case report, as determined by fluorescence-activated cell sorter (FACS) analysis of propidium iodide-stained semen samples (Vicari et al., 2002). Lalonde et al. (1988) investigated whether the absence of acrosomal components influenced nuclear chromatin decondensation. Globozoospermic sperm cells were therefore exposed to either dithiothreitol (DTT) or EDTA, which are known to induce nuclear chromatin decondensation. No difference was found between globozoospermic and normal spermatozoa (Lalonde et al., 1988). In contrast, Carrell et al. (1999) showed an increased decondensation rate in two affected siblings when exposed to heparin, from which they concluded that chromatin is less stable in globozoospermia (Carrell et al., 1999).

Taken together, these findings indicate that chromatin condensation is disturbed in globozoospermia, with a high heterogeneity in the degree of maturity. The aetiology of these findings remains to be revealed.

DNA fragmentation

DNA strand breaks can be visualized by labelling the 3'-OH ends using the TdT (terminal deoxynucleotidyl transferase)-mediated dUTP-biotin nick-end labelling (TUNEL) assay, which is indicative of DNA fragmentation. Both Baccetti et al. (1996) and Vicari et al. (2002) found an increased percentage of DNA fragmentation in globozoospermic sperm cells compared with fertile controls (10 versus 0.1% and 37 versus 22.5%, respectively). The neutral single-cell gel electrophoresis or COMET assay also quantifies double- stranded DNA breaks. Larson et al. (2001) found no elevation of DNA fragmentation in round-headed sperm cells compared with fertile controls, which was in accordance with their SCSA results, described in the previous section.

The sperm-ubiquitin tag immunoassay (SUTI), which detects DNA damage as well, was developed to identify abnormal sperm cells (Sutovsky et al., 2001). Round-headed sperm cells were highly ubiquinated, indicating defective DNA.

Cytogenetics of sperm cells

Whether morphological sperm deformities are linked to sperm cell chromosomal abnormalities has been investigated extensively but is still controversial. Carrell et al. (1999) published two case reports in which siblings with globozoospermia were evaluated by fluorescence in-situ hybridization (FISH). After the evaluation of >5000 cells, they found increased aneuploidy rates for chromosomes 13, 21 and XY in one of two affected siblings and a mildly increased aneuploidy rate for chromosome 21 in the other sibling (Carrell et al., 1999). In a similar study, three siblings, of whom two brothers were affected, were tested. These authors found no significant increase in aneuploidy rates for chromosomes 18, X and Y in either sibling but did detect a significant increase in chromosome 15 aneuploidy in one of the affected siblings (4.03 versus <0.4% in fertile controls) and his fertile brother (1.18%). The other affected brother, however, did not show a significant increase in aneuploidy rates (Carrell *et al*., 2001). Martin *et al*. (2003) also tested chromosomes 15, X and Y in over 10,000 cells. They did not detect an elevated rate of chromosome 15 aneuploidy but did report an increase in XY disomy (Martin et al., 2003). Recently, these findings were supplemented with the observation of increased aneuploidy rates of chromosomes 13, 16 and 21 in a globozoospermic man compared to a normozoospermic man (Ditzel et al., 2005). Other studies could not confirm a higher aneuploidy rate in globozoospermic cells compared with aneuploidy rates in sperm cells of fertile controls (Rybouchkin et al., 1996; Viville et al., 2000; Vicari et al., 2002; Morel et al., 2004), although Morel et al. did observe a significantly higher disomy rate of chromosomes 13 and 21 in one patient compared with another globozoospermic man. Machev et al. (2005) have reviewed these papers, who concluded that increased aneuploidy rates occurred mostly in

the acrocentric (13, 14, 15, 18 and 21) and sex chromosomes and that these findings do not differ from other types of infertility.

Genetic etiology?

A genetic basis for globozoospermia was suspected and is supported by several case reports of families with two or more affected siblings (Kullander and Rausing, 1975; Florke-Gerloff et al., 1984; Dale et al., 1994; Carrell et al., 1999, 2001; Kilani et al.,2004). The mode of inheritance remains obscure. By investigating the occurrence of sperm defects in consanguinity, no additive evidence was found for an autosomal recessive condition in case of globozoospermia (Baccetti et al., 2001). Also, evidence for the participation of Y chromosome microdeletions in globozoospermia could not be established (Gunalp et al., 2001). As early as 1975, Moutschen and Colizzi designated acrosomelessness as an interesting and efficient tool in mammalian mutation research. Up to now, four mouse genes have been associated with globozoospermia. The first gene, the so-called *blind-sterile* (*bs*) mutation, was identified in 1986. This was an autosomal recessive mutation on chromosome 2 that was shown to result in a failure to assemble an acrosome, a severely impaired spermatogenesis leading to a strongly reduced sperm count, and absence of motility and bilenticular cataract (Sotomayor and Handel, 1986).

In a more recent publication, globozoospermia was reported in mice with a homozygous deletion of the *Csnk2a2* gene[1]* (Xu et al., 1999; Rocha and Affara, 2000; Truong et al., 2003). This gene encodes a substrate of protein kinase casein kinase II, which is preferentially expressed in the late stages of spermatogenesis and is associated with the nuclear matrix. In these mice, the nucleus of spermatozoa was deformed. In spermatids, the acrosome often detached from the nucleus and disappeared during the next stages of spermatogenesis. Also, oligozoospermia due to Sertoli cell phagocytosis and apoptosis was observed (Xu et al., 1999). Recently, the *CSNK2A2** gene has been examined for mutations in human globozoospermia patients. Despite expectations, no mutations were detected in any of six investigated patients (Pirrello et al., 2005).

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* Literature searches in another context revealed an article (Escalier, Silvius *et al*., 2003), in which the authors of the same group that discovered the relation between *Csnk2A2* and globozoospermia (Xu, Toselli *et al*., 1999) concluded in this paper that their former conclusions on *Cnsk2A2*$^{-/-}$ mouse sperm cells were incorrect regarding the morphology characteristics. Although these sperm cells appeared round-headed by scanning electron microscopy, closer examinations revealed poorly elongated nuclei and anomalies, but not absence of the acrosome. They explain these misconceptions by the presence of a coiled flagellum around the nucleus and a cytoplasmic droplet looking like a round nucleus. They clearly state that this phenotype is different from (total) human globozoospermia. This could contribute to the fact that no mutations were found in human globozoospermia patients.

Globozoospermia has also been described in *Hrb*$^{-/-}$ mice. Besides the characteristic round-headed and acrosomeless sperm cells, also sperm cells with a midpiece lacking a mitochondrial sheath in combination with reduced sperm motility were observed (Kang-Decker et al., 2001). Additional characteristics included loss in cell polarity, intracellular flagellar coiling, multinucleation, supernumerary centrioles and multiflagellation and nuclear vacuolization (Juneja and Van Deursen, 2005). The *Hrb* gene encodes for the HIV-1 Rev binding protein, an important factor in proacrosomic vesicle fusion, which is abundantly transcribed during spermiogenesis. As a consequence, proacrosomic vesicles are formed in the medulla of the Golgi apparatus in *Hrb*$^{-/-}$ mice spermatids but fail to fuse and transform into an acrosome (Kang-Decker et al., 2001). Kierszenbaum et al. (2004) investigated the factors involved in sperm nuclear morphogenesis and identified a structure they previously designated the acroplaxome. This cytoskeletal scaffold, which, amongst others, contains F-actin and keratin 5, develops in the subacrosomal space and anchors the developing acrosome to the nuclear envelope and is involved in acrosome formation. When they studied the acroplaxome in *Hrb*$^{-/-}$ mice, they observed a deficiency in keratin 5 and the formation of a pseudo- acrosome (Kierszenbaum et al., 2004). The latter result was definitely reminiscent of the observations described by Baccetti et al. (1977), which would make Hrb an interesting candidate gene for human globozoospermia. Unfortunately, the first results on human globozoospermia patients are yet less promising (Christensen et al., 2006).

The most recently described mouse gene that is known to result in globozoospermia when knocked out is called the Golgi-associated PDZ and coiled-coil motif containing protein (*Gopc*) gene[2#]. In spermatids, this protein is localized in the trans-Golgi cisternae and the trans-Golgi network. It plays a role in Golgi-to-membrane vesicle transport. In early spermatids, the fragmentation of the acrosomal cap was observed, as well as abnormal proacrosomic vesicles that failed to fuse (Yao et al., 2002).

Characteristics that remind of the observations made in human globozoospermia patients in early reports (Baccetti et al., 1977; Castellani et al., 1978; Nistal and Paniagua, 1978; Florke-Gerloff et al., 1985) were observed in later studies. These concluded that in *Gopc*$^{-/-}$ mice larger proacrosomic vesicles are formed and do attach to the poorly developed acroplaxome but that the vesicles detach and are lost when the spermatozoon is released into the lumen. Multiple

PDZ domains were originally identified in the post-synaptic density protein PSD-95 as three repeats of about 90 residues containing the conserved motif Gly-Leu-Gly-Phe (GLGF) 3. The name PDZ domain is derived from the names of three proteins containing such domains [PSD-95, the *Drosophila* discs-large tumor suppressor protein DlgA and the tight junction protein ZO-1]; alternative designations are GLGF-repeat and DIgA homology region, DHR (Saras and Heldin, 1996).

Gene	Encoding protein	Chromosome	Human homologue	Reference
Bs–/–, blind-sterile	?	2	–	Sotomayor and Handel (1986)
Csnk2a2–/–	Casein kinase II (Ck2)	8	16 (p13.3–p13.2), 56 749 315–56 789 283	Xu *et al.*, (1999), Escalier *et al.* (2003), Truong *et al.* (2003), Pirrello *et al.* (2005)
Hrb–/–	HIV-1 Rev binding protein	1	2 (q36.3), 228 162 547–228 247 805	Kierszenbaum *et al.* (2004), Kang-Decker *et al.* (2001), Juneja and Van Deursen (2005)
GOPC–/–	Golgi-associated PDZ and coiled–coil motif containing protein	10	6 (q21), 117 988 128–118 030 374	Yao et al. (2002), Suzuki-Toyota *et al.* (2004), Ito *et al.* (2004)

Table III Globozoospermia in mouse models.

Human homologues found at http://www.informatics.jax.org/.

failures regarding the nucleus, caudal manchette, post-acrosomal sheath and posterior ring were noted, resulting in impaired spermatid nuclear elongation (Ito et al., 2004). In detail, the defective posterior ring did not influence tail formation, but during epididymal passage, the tail was coiled around the nucleus, with the dislocation of the implantation fossa and a disorganized mitochondrial sheath (Suzuki-Toyota et al., 2004).

The last three candidate genes (Table III) have recently been screened in two men with (partial) globozoospermia. This study led to the identification of polymorphisms, but no mutation with a clear link to partial globozoospermia was found (Christensen et al., 2006). These results have yet to be confirmed in a larger group of patients with partial or total globozoospermia, before conclusions on these candidate genes can be drawn. Undoubtedly, the identification of proteins and protein complexes that are involved in spermiogenesis will substantially facilitate the search for additional candidate genes (Luo et al., 2003).

CONCLUSIONS AND DISCUSSION

Globozoospermia can be diagnosed by an extended semen analysis, which is likely to show the typical feature of round-headed, acrosomeless spermatozoa. A slight asthenozoospermia might also be found. No peculiarities in case history or physical examination of affected males have been associated with globozoospermia. The pathogenesis occurs during spermiogenesis and probably originates in acrosomic vesicle fusion and cytoskeleton disorders, which remains to be elucidated. Genetically manipulated mice show that the knockout of different genes can lead to approximately the same phenotype. Moreover, these observations show similarities with the results reported several decades ago (Baccetti et al., 1977; Castellani et al., 1978; Nistal and Paniagua, 1978; Florke-Gerloff et al., 1985), which indicates that there might indeed be different genetic pathways of pathogenesis that lead to this sperm morphology disorder (Sotomayor and Handel, 1986; Xu et al., 1999; Kang-Decker et al., 2001; Yao et al., 2002; Kierszenbaum et al., 2004).

This could also explain the variable results in the cases of globozoospermia as described above. It is clear that a Schirren-Holstein or type I globozoospermia is a common term for 100% of round-headed and acrosomeless spermatozoa per ejaculate. The term type II globozoospermia for round-headed spermatozoa with an acrosome but with maturation defects was not broadly adopted in literature (Anton-Lamprecht et al., 1976; Singh, 1992; Christensen et al., 2006). Also, the use of the current nomenclature, namely type I and II, is not consistent and therefore misleading, as well in literature as in daily practice. We therefore

suggest replacing these terms by total globozoospermia in case of 100% round-headed, acrosomeless spermatozoa and of partial globozoospermia if <100% of the spermatozoa are affected. Because globozoospermia is a rare disorder, only a relatively small number of patients have been described in literature. Moreover, various methods have been used to study these patients. These factors make an overall analysis very difficult. In our opinion, systematic collection and citation of all the facts found was the best solution, severe selection on quality would have excluded too much information. This implicates that no firm conclusions can be drawn. Instead, an overview of what has been written on globozoospermia in the past three decades has been provided to outline the syndrome globozoospermia.

Next to these academical considerations, physicians in their daily practice are in need of treatment options for these patients. No treatment was available until ICSI was introduced, which seemed to be a promising solution. Unfortunately, fertilization rates after ICSI often appear to be severely diminished in globozoospermia (Table II). A solution to this problem could be to use diagnostic heterologous ICSI to evaluate the fertilization capacity for each case, in order to predict the need of oocyte activation with a calcium ionophore (Heindryckx et al., 2005).

An important factor to consider is the possible effect on offspring of the abnormalities in chromatin structure and DNA integrity that, although inconsistently, have been described in globozoospermic cells. Whenever possible, these factors should be evaluated for each case in specialized centres. Obviously, preferably normally shaped sperm cells should be used in case of partial globozoospermia.

A lot of questions on globozoospermia remain to be answered. Additional studies are needed to elucidate whether cases of partial globozoospermia and total globozoospermia are variations of the same syndrome. Genetic analysis could be of great distinctive value in this matter, although environmental factors involved in globozoospermia etiology cannot be ruled out completely. Nevertheless, genetic analysis in globozoospermia could be easier than in male subfertility in general, because of its distinct morphological characteristics, which places its origin in spermiogenesis.

Therefore, studies should be focused on genes that are involved in the Golgi apparatus and cytoskeleton structures during spermiogenesis, like the candidate genes found in mice. Although mouse models may not be directly applicable to the human situation, these models may nevertheless help us better understand mammal spermatogenesis, giving us a perspective on the cause(s) of human globozoospermia. The fact that similar pathophysiologies are found in both mice and men does not necessarily mean that the homologous genes are involved

in both species but surely gives an indication on what kind of genes we are looking for. In addition, the rarity of the syndrome is suggestive for a homozygous inheritance pattern, although Baccetti et al. (2001) did not find correlation between consanguinity and globozoospermia. Candidate genes could be identified by linkage analysis in consanguineous families, or families with more than one affected member, as the syndrome is too rare to occur in first-degree family members by chance. Also, the identification of proteins that are involved in spermiogenesis and the etiology of globozoospermia by testing testis mRNA on expression microarrays could provide candidate genes that could subsequently be screened in patients and their families.

In conclusion, genetic research on globozoospermia is still in its initial stages but will undoubtedly prove to be invaluable in elucidating the processes of spermiogenesis and spermatogenesis in general and of the etiology of globozoospermia in particular.

Acknowledgements
The authors thank Leonie van den Hoven and Hannie Robben for their expert technical assistance and Henry Dijkman for his expertise on electron microscopy and the provided images.

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Part 2

Partial globozoospermia

Chapter 3

Morphology of partial globozoospermia

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J Androl, 2010. 32(2): p 199-206

ABSTRACT

Total globozoospermia is a rare sperm morphology disorder that consists of 100% round-headed, acrosomeless spermatozoa. There is also a larger group of patients whose sperm cells are partially acrosomeless. The aim of this investigation was to describe partial globozoospermia compared to total globozoospermia and normozoospermia. Ejaculates from 10 patients with more than 50% acrosomeless spermatozoa (partial globozoospermia), 3 patients with total globozoospermia, and 9 normozoospermic controls were analyzed with light microscopy, transmission electron microscopy, and flow cytometry. Qualitative and quantitative examination of spermatozoa from the 3 groups shows differences in the percentage of round-headed sperm cells and acrosome malformation. Total globozoospermia presents as a homogenous kind of teratozoospermia. Partial globozoospermia is a distinctive sperm malformation with an increased proportion of round-headed sperm cells and acrosome malformations compared to normozoospermia, which exists separately from total globozoospermia. It thereby contains oval sperm cells that may have distinctive malformations of the sperm head matrix, but also morphologically normal sperm cells that may be used in a clinical setting.

Key words: Sperm morphology disorder, male subfertility.

INTRODUCTION

Total globozoospermia is a well-described sperm morphology disorder that consists of round-headed, acrosomeless spermatozoa (Schirren et al, 1971). Men suffering from this syndrome are considered infertile, although the introduction of intracytoplasmic sperm injection (ICSI) has made some pregnancies possible in this patient group (Larson et al, 2001). Given its morphological features, globozoospermia likely originates during spermiogenesis. This theory has been confirmed in several mouse knockout models (Kang-Decker et al, 2001; Yao et al, 2002; Xiao et al, 2009). A candidate gene has been identified in humans. The mutation in *SPATA16* indicates a genetic origin in human spermiogenesis as well, but we found no mutations in other patients with globozoospermia except the one in the affected family (Dam et al, 2007b). These findings reinforce the idea of a multigenetic disorder in which several etiologies may cause the same morphologic phenotype.

There is a group of patients whose semen samples show only a fraction with this typical head shape. The research group that first described globozoospermia noted this ''partial'' condition only two years after their initial publication (Holstein et al, 1973). In the first description of partial globozoospermia, the round-headedness was attributed to a cytoplasmic droplet surrounding the sperm head and acrosome. The authors named it type II globozoospermia (Anton-Lamprecht et al, 1976). This nomenclature was rarely used in the literature, and when it was, it referred to partially affected individuals who did not have the other characteristics that Anton-Lamprecht and colleagues (1976) described earlier. Because of this confusion in nomenclature, we suggest the terms ''total'' and ''partial'' globozoospermia (Dam et al, 2007a).

Cases with a large proportion of acrosomeless spermatozoa are more common in daily clinical practice than total globozoospermia. Although there is no defined threshold for the normal proportion of round- headed spermatozoa in an ejaculate, it is well known that even fertile men have up to 3% sperm cells in the ejaculate (Kalahanis et al, 2002). Unfortunately, many reports describe cases of partial globo- zoospermia without detailed morphology studies using, for example, transmission electron microscopy (TEM) providing validation of this condition, which leads to confusing results (Holstein et al, 1973; Pedersen and Rebbe, 1974; Weissenberg et al, 1983; Florke-Gerloff et al, 1984; Syms et al, 1984; Tyler et al, 1985; Singer et al, 1986; Lanzendorf et al, 1988; Rybouchkin et al, 1996; Carrell et al, 1999, 2001; Coetzee et al, 2001; Larson et al, 2001; Christensen et al, 2006). No detailed morphological description has yet been provided for cases of partial globozoospermia. The main purpose of this study was to investigate whether partial globozoospermia and total globozoospermia are

two different morphology disorders and to describe sperm characteristics in partial globozoospermia.

MATERIALS AND METHODS

Design

We included 10 patients who had more than 50% acrosomeless spermatozoa as the main morphologic head characteristic (partial globozoospermia) and compared their spermatozoa with the spermatozoa of three patients with total globozoospermia and nine normozoospermic controls. We used qualitative techniques such as Diff-Quik staining, TEM, and quantitative flow cytometry to determine proacrosin and acrosin levels for this purpose.

Patients

Patients were recruited at the Centre for Reproductive Medicine in Nijmegen, The Netherlands. Infertile men (as evidenced by ≥ 1 year of unprotected intercourse) are routinely subjected to a semen analysis, including sperm count and motility assessment in accordance with World Health Organization (WHO) guidelines. In our center, the morphology is routinely assessed with aniline blue and eosin-Y staining (Ebisch et al, 2006), and we used this staining method for patient inclusion. Briefly, several drops of the semen sample are incubated for 10 minutes at room temperature with trypsin, mixed with 2% eosin and 25% aniline blue stain on a glass slide, smeared out, and air dried. The slides show a blue background, vital sperm cells stain white, and nonvital sperm cells stain red. We used strict criteria for the morphology evaluation of the vital cells (Menkveld et al, 2001). Sperm head morphology is considered normal if there is an oval head, a smooth surface, and an acrosome that covers 40%–70% of the head. Two trained technicians evaluated at least 200 sperm cells per sample. Patients whose ejaculate had more than 50% acrosomeless sperm cells were invited to participate in the study. Controls were selected from among normozoospermic men who were enrolled in an in vitro fertilization (IVF) program because of a female factor. Total globozoospermia is rare, so that the three patients who are included in this study received treatment at the Egyptian IVF-ET Center in Cairo. This study was approved by the regional Ethics Committee in Nijmegen. All patients gave informed consent for the study.

Each of the participants provided a second fresh sample for the study. These fresh samples were processed for standard semen analysis (WHO, 1999), including a Diff-Quik stain and TEM for morphology evaluation. The remnants of the samples were then frozen for future use in flow cytometry analysis.

Diff-Quik Stain

We evaluated the morphology with Diff-Quik stain only for the second fresh sample as an alternative to Papanicolaou stain (WHO, 1999). In short, a drop of semen was smeared on a slide and air dried. We stained the slides automatically with the Wescor 7120 Aerospray Hematology Slide Stainer (Elitech Group, Puteaux, France) for staining with the reagents eosin and thiazin (azure B and methylene blue). Two technicians evaluated at least 200 cells per patient sample at 61000x magnification. The main outcome terms used to describe the sperm head morphology were normal (or oval), dysmorphic, and round. The acrosome was scored as present or absent.

Transmission Electron Microscopy

For electron microscopy, each sample was diluted or concentrated to a final sperm concentration of 10–25 x 10^6 sperm/mL (10 x 10^6 sperm/pellet). Sperm cells were washed and concentrated by centrifugation in human tubal fluid medium, supernatant was discarded, and the pellet was fixed in 2.5% glutaraldehyde left to dissolve in 0.1 M sodium cacodylate buffer, pH 7.4, at 4°C overnight. Subsequently, samples were washed in the same buffer, postfixed in Palade buffered 2% OsO_4 for 2 h, pelleted in 15% gelatin, dehydrated, and embedded in Epon 812 (Merck, Darmstadt, Germany). Ultrathin sections were sliced on an ultratome (Leica, Reichert Ultracuts, Vienna, Austria), contrasted with 4% uranyl acetate for 45 minutes and then with lead citrate for 5 minutes at room temperature. Sections were examined in a Jeol 1200 EX2 electron microscope (JEOL, Tokyo, Japan). Only cells with a complete sperm head and at least one-third of the tail (including midpiece) were included for evaluation. Thirteen to 30 cells per field were found for each patient. In total, 189 cells were evaluated in normozoospermia, 213 in partial globozoospermia, and 54 in total globozoospermia. Morpho- logic outcomes evaluated are based on previous literature descriptive features (normal or aberrant), sperm head morphology (oval or round), acrosome presence (yes, partially, or absent), chromatin condensation (highly or poorly condensed), invagination (no or yes), mitochondria in the sperm head (no or yes), and space between the nuclear membranes (no or yes; for review see Dam, 2007a).

Flow Cytometry for Acrosin and Proacrosin

Immunofluorescent Staining frozen-thawed sperm samples were washed twice in phosphate-buffered saline (PBS), fixed in 1% paraformaldehyde (15 minutes), and preincubated in 10% normal goat serum (NGS, 10 minutes). An aliquot of each sample was held aside for the base (background) measurement. The rest of the sample was then incubated for 1 h at room temperature in either mouse

proacrosin monoclonal antibody (mAb) 4D4 (Escalier et al, 1991), 1% (dilution 1:100 PBS:10% NGS) or mouse acrosin mAb Acr-C5F10 (Biosonda, Santiago, Chile) 2.5% (dilution 1:40 PBS:10% NGS). Samples were then washed in PBS before the second incubation with 0.2% Alexa 488 goat anti-mouse (dilution 1:500 PBS:10% NGS; Molecular Probes, Eugene, Oregon) for 1 hour at room temperature in the dark. Then the samples were washed in PBS and brought to a final volume of 500 uL for the flow cytometry. After flow cytometry measurements, the samples were counterstained with 100 mL of 4,6-diamino-2-phenylindole (0.0025 mg/mL; Sigma, St Louis, Missouri) and incubated for 10 minutes. A droplet of this sperm suspension was dried on a glass slide to check the staining pattern. The slides were mounted in 20 mL Vectashield (Vectra Laboratories, Burlingame, Califormia) to preserve staining; the sperm samples were photographed under a fluorescence microscope at magnification 6400–6630.

Flow Cytometry—At least 2000 cells per sample were analyzed on a Coulter Epics Elite flow cytometer (Beckman Coulter, Miami, Florida) equipped with a 488-nm excitation laser (for the presence and intensity measurements of proacrosin and acrosin). After background correction, using an unstained sample, proacrosin or acrosin intensity was measured on photomultiplier tube 2 log (525 bp 615 nm) and gated on forward scatter vs side scatter to discriminate cells from debris. The main outcomes were the percentages of gated cells positive for acrosin and proacrosin staining and the mean staining intensity ($\bar{x}$) on a logarithmic scale.

Statistical Analysis

Results of aniline blue and eosin-Y staining were compared with Diff-Quik staining in a separate paired samples t-test for each patient group. The percentages of normal patterns in TEM in normozoospermia and partial globozoospermia and acrosome presence per morphology category were compared with the X^2 test. All other results were analyzed with 1-way analysis of variance (ANOVA) along with the Welch and Brown-Forsythe tests of robustness, and the Tukey, Tukey-B, and Games-Howell tests as post hoc tests. $P < .05$ was considered significant. We used the SPPS 15.0 software pack for statistical analysis.

RESULTS

Table 1 shows the results of the routine semen analyses and the summarized results for the Diff-Quik staining of the second samples of all patients and controls. Although the inclusion criterion for the partial globozoospermia group was the presence of more than 50% acrosomeless sperm, the acrosomeless sperm

	Volume (range)	Concentration (range)	Motility (range)	% Morphology (range)	% acrosomeless
Controls	3.4 (1.9-6.3)	56 (25-85)	55 (25-85)	81 (70-95)	0
Partial globozoospermia	2.9 (0.8-5.0)	21 (4-50)	22 (10-35)	98 (95-100)	58 (30-82)
Total globozoospermia	2.0 (1.0-3.0)	70 (70)	38 (25-50)	100 (100)	100 (100)

Table 1 Semen analysis.

The mean values and percentages of the different parameters in semen analysis are displayed. Volume is expressed in ml; concentration is expressed in million spermcells/ ml; motility represents progressive motility (A+B), morphology represents abnormal head morphology and acrosomeless is the percentage of acrosomeless spermatozoa. Morphology analysis was performed by aniline/ eosin Y staining and may show somewhat different results than the Diff-Quik staining.

in the second sample varied from 30% to 82% with aniline blue and eosin-Y staining and between 25% and 83% in the Diff-Quik staining. The semen volumes of the three groups were comparable. The sperm concentrations of patients with partial globozoospermia were significantly lower than those of the controls and the patients with total globozoospermia ($P = .002$ and $P < .001$, respectively), whereas motility was only significantly different between controls and patients with partial globozoospermia ($P = .002$). On the basis of the inclusion criteria used in this study, the proportions of acrosomeless sperm obviously differ in the three groups. Aniline blue and eosin-Y staining and Diff-Quik staining correlated well with respect to morphology and acrosome absence in partial and total globozoospermia, but differed significantly with respect to the percentage of normal morphology (19% vs 13%, P = .031) and acrosome absence of the controls (0% vs 5%, P = .022).

Table 2 displays the detailed morphologic evaluations of the Diff-Quik staining. Oval head forms (normal sperm cells) are rarely found in partial globozoospermia (mean 1%), and never in total globozoospermia. The absence of the acrosome is rarely seen in normozoospermia (mean 5%; see Table 1), but is the predominant morphologic characteristic in total globozoospermia. In partial globozoospermia, we found a mean of 53% acrosomeless cells (see Table 1). Acrosomeless cells were present in the dysmorphic sperm cells (mean 40%), but mostly in round-headed cells (mean 93%; see Table 2). We found a significant

Morphology Group	Sample Type	Spermatozoa in Specific Sperm Head Morphology Group[b]	*P*	% Acrosomeless[c]	*P*
Oval heads	Normozoospermia	13 (5–17)		0	
	Partial globozoospermia	1 (0–4)	<0.001[d]	0	
	Total globozoospermia	0		. . .	
Dysmorphic heads	Normozoospermia	85 (81–94)		4 (0–15)	
	Partial globozoospermia	74 (53–93)	<0.001[d]	40 (19–77)	<0.001[d]
	Total globozoospermia	3 (0–7)		100	
Round heads	Normozoospermia	2 (0–5)		58 (25–100)	
	Partial globozoospermia	25 (7–47)	<0.001[d]	93 (68–100)	<0.001[d]
	Total globozoospermia	97 (94–99)		100	

Table 2 Morphological characteristics of sperm with Diff-Quik staining [a].

[a] The mean percentages (with ranges in parentheses) are shown for each specific head morphology in each patient group related to the presence of acrosome found after Diff-Quik staining.

[b] The distributions of sperm types in the categories normal, dysmorphic, and round-head morphology are displayed by patient group.

[c] The percentages of sperm without acrosomes are given by head morphology characteristics in each patient group.

[d] Significantly different with respect to normozoospermia and total globozoospermia.

difference between these morphological head characteristics and the presence of acrosomeless cells in all groups ($P < .001$).

Figure 1 displays a TEM picture of a representative cell for each of the 3 groups. The results for TEM were analyzed with 1-way ANOVA, which showed a significant difference ($P < .001$) between groups for all features. In detail, no significant difference for chromatin condensation was found between normozoospermia and partial globozoospermia. Invagination, mitochondria in the sperm head, and space between membranes differed nonsignificantly between total and partial globozoospermia.

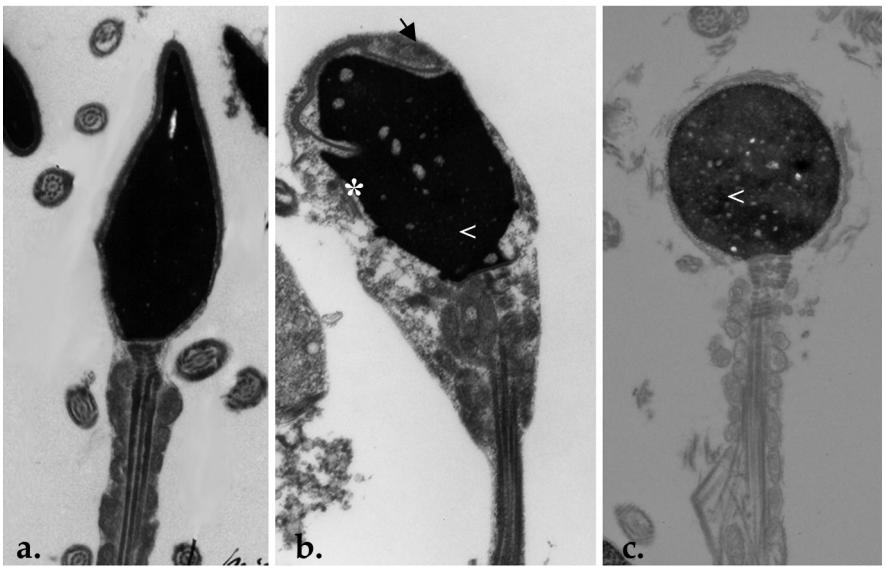

Figure 1a-c TEM pictures.

a normozoospermia - all organelles are present and organized as a normal sperm cell; **b** partial globozoospermia – a more or less oval head shape with less condensed chromatin (<<), a partially present acrosome. and a disorganized midpiece. Mitochondriae (➔) are present not only in the midpiece, but also in the sperm head. Invagination of the acrosome (*) is an often observed abnormality, and the acrosome is also incorrectly attached; **c** the round-headed, acrosomeless form, which is characteristic for total globozoospermia

Table 3 displays all TEM characteristics related to the 2 main types of sperm head morphology (round and oval). The amounts of oval and round sperm cells were significantly different between normozoospermia, partial globozoospermia, and total globozoospermia. When sperm head characteristics were analyzed

Sperm head morphology group	Sample type	Nr. of sperm analyzed	sperm head morphology type (%)		% Acrosome[a]			
					absent		partial	
			(range)	*p*	*(range)*	*p*	*(range)*	*p*
Oval headed	Normozoosp.	176	93 *(71–100)*		13 *(0–24)*		1 *(0–8)*	
	Partial globozoosp.	146	68 *(8–89)*	**0.04***	7 *(0–17)*	0.95	31 *(9–100)*	**<0.001***
	Total globozoosp.	2	4 *(0–7)*	<0.001#	50 *(0–100)*	-	0 .	-
Round headed	Normozoosp.	13	7 *(0–29)*		36 *(0–100)*		0 .	
	Partial globozoosp.	67	32 *(11–92)*	**0.04***	18 *(0–29)*	0.98	46 *(0–80)*	**0.002***
	Total globozoosp.	52	96 *(96–100)*	<0.001#	32 *(15–46)*	0.46	27 *(9–46)*	0.12

Table 3 Sperm head morphology and structures observed by TEM [a].

Abbreviation: TEM, transmission electron microscopy.

[a] This table displays the mean percentages (with ranges in parentheses) for the sperm head morphology and specific structures found in sperm as observed with TEM. P values are displayed for partial globozoospermia with respect to normozoospermia and for total globozoospermia in respect to partial globozoospermia. Significantly different values for partial globozoospermia are in bold.

[b] Mean percentage of cells per patient either acrosomeless or with a partially present acrosome according to sperm head morphology.

[c] Mean percentage of cells per patient with poorly condensed chromatin according to sperm head morphology.

within the oval sperm cells, considered normal in this setting, partial globozoospermia differed significantly from normozoospermia for partially formed acrosomes ($P < .001$), mitochondria in the sperm head ($P < .001$), and space between the sperm head membranes ($P < .001$). In the outer right column of Table 3 are the percentages sperm cells with normal characteristics (presence of acrosomes, highly condensed chromatin, no invagination or mitochondria in the sperm head, and no space between sperm head membranes). These percentages differed significantly for controls and patients with partial globozoo-

% poor chromatin[b]		% Mitochondria[c]		% Invagination[d]		% Membrane space[e]		% Total normal patterns[f]	
(range)	*p*	*(range)*	*p*	*(range)*	*p*	*(range)*	*p*	*(range)*	*p*
33 *(8–52)*		1 *(0–8)*		2 *(0–11)*		27 *(16–50)*		*40* *(22–58)*	
39 *(0–73)*	0.50	12 *(0–50)*	**<0.001***	7 *(0–100)*	0.07	50 *(44–100)*	**<0.001***	*17* *(0–32)*	**0.004***
50 *(0–100)*	-	0 .	-	0 .	-	50 *(0–100)*	-	*0* .	
69 *(40–100)*		0 .		0 .		31 *(0–67)*		No normal patterns present	
63 *(0–86)*	0.89	18 *(0–60)*	**0.01***	8 *(0–73)*	**0.01***	57 *(0–86)*	0.21		
81 *(73–94)*	0.71	13 *(4–23)*	0.79	8 *(6–10)*	0.20	52 *(32–71)*	0.86		

[d] Mean percentage of cells per patient with mitochondria in the sperm head according to sperm head morphology.
[e] Mean percentage of cells per patient with invagination of the acrosome in the sperm head according to sperm head morphology.
[f] Mean percentage of cells per patient with a space between the sperm head membranes according to sperm head morphology.
[g] Mean percentage of cells presenting normal head morphology features in TEM.
[h] Significantly different with respect to normozoospermia.
i Significantly different with respect to partial globozoospermia.

spermia (P = 0 .004) in the results of the X^2 test. Invagination was not significantly different (P = 0.07). Chromatin condensation did not differ significantly in the 3 sperm sample types. In the total globozoospermia group only two oval sperm heads were found; therefore, no conclusions about the oval forms of this group can be reached.

Round-headed sperm cells in partial globozoospermia differed significantly from those in normozoospermia for partially formed acrosomes (P = .002),

mitochondria in the sperm head (P = .01), and invagination (P = .01). There were no significant differences for acrosome absence, chromatin condensation, or the space between head membranes. There was a significant difference in mitochondria in the sperm head between controls and total globozoospermia (P = .019; not shown in Table 3). It is noteworthy that in this setting all the observed features in the round-headed cells were comparable between partial and total globozoospermia.

Figure 2 shows the flow cytometry measurements of the acrosin and proacrosin; the mean intensities of all samples for the proacrosin and acrosin staining are given on the X-axis and the percentage of positive cells on the Y-axis. Unfortunately, control 9 was lost for analysis. The mean cell positivity and staining intensity show a differential pattern for each group: in total globozoospermia a small percentage of the sperm samples stained with low intensity for proacrosin and acrosin. The percentage of positive cells in partial globozoo-

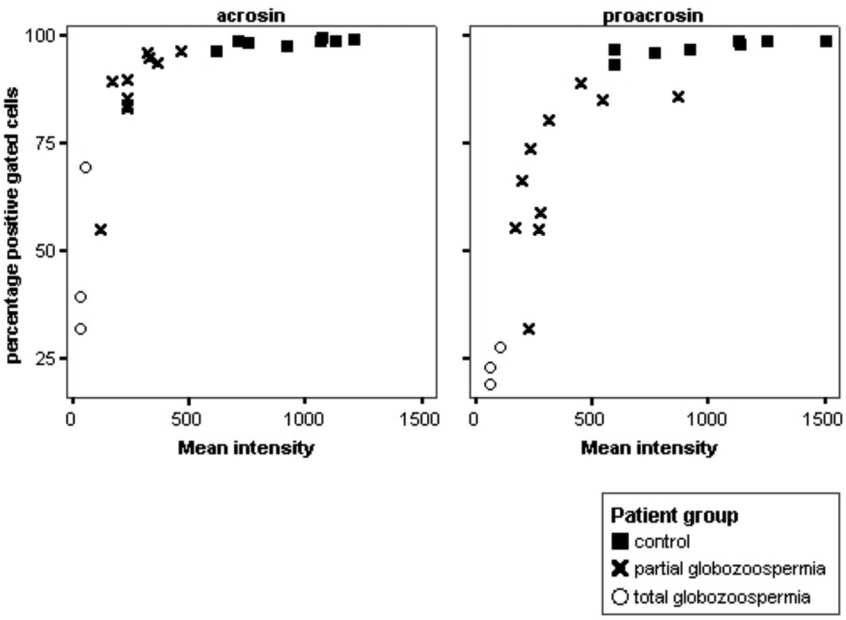

Figure 2 Flow cytometry results after proacrosin and acrosin staining.

Percentages of gated cells that are positive for acrosin and proacrosin are displayed on the x-axis. The results of the intensity measurements (arbitrary units) using a monoclonal antibody for acrosin or proacrosin are presented on the y-axis. The 3 groups present different staining patterns (content) for both acrosin and proacrosin. In partial globozoospermia, the acrosomes are present, but show a low intensity, which indicates little activity.

spermia samples was about equal to that in control samples in acrosin staining. The acrosin staining intensity in partial globozoospermia was intermediate between the staining intensities of total globozoospermia samples and control samples, suggesting a partially present or malformed acrosome.

In proacrosin staining, a lower percentage of positive cells and a far lower intensity were found for partial globozoospermia in comparison to controls. Statistical analysis showed significant differences between the groups in both the mean intensity of staining ($P = .001$) and numbers of positive cells ($P = .002$).

DISCUSSION

As a result of this study, we would describe partial globozoospermia as an oligo-asthenoteratozoospermia that contains an increased amount of round-headed sperm cells (>25% in this study) as well as an increased occurrence of acrosomes defects (>25% in this study). Detailed observation by TEM shows an occurrence of differentiation defects in oval sperm heads that is significantly different from that of normozoospermia, next to a limited number of normal sperm cells that might be used in ART (assisted reproductive techniques).

Both partial and total globozoospermia are rare conditions and therefore difficult to investigate. However, such investigations of these conditions will improve the further knowledge of this specific type of sperm morphology; characteristics of these types of sperm morphology aberrations facilitate the classification of patients' conditions, reveal information about their prognoses in fertility care, and unravel the details of a potential abnormal mechanism during spermatogenesis. As for the robustness of our results, we were hampered by the small number of patients available for participation, which makes analysis and interpretation more difficult. Thereby, we used techniques that are common in our laboratory, such as aniline blue and eosin-Y staining, to select our patients. We added the Diff-Quik stain to adhere to WHO (1999) standards. The results of aniline blue and eosin-Y staining and Diff-Quik staining did correlate well for morphology and acrosome presence in partial and total globozoospermia, but differed significantly for morphology and acrosome absence in the control samples. This finding might be due to the fact that in the aniline blue and eosin-Y staining only vital cells are assessed. As for the staining of the acrosome, in spite of the fact that only acrosin antibodies are commercially available, but we were able to obtain a proacrosin antibody (kind gift of Dr. D. Escalier, Paris). Although it is assumed that in nonactivated sperm only proacrosin is present and that it converts to acrosin after capacitation and acrosome reaction, both proacrosin and acrosin are present in human ejaculated sperm (Florke-Gerloff et al, 1984;

Jeyendran et al, 1985; Lalonde et al, 1988). Our results support these findings.

In this experimental setting, we selected partial globozoospermia patients with the criterion of more than 50% acrosomeless sperm cells. We found a variation of the numbers of acrosomeless sperm cells between the first and second samples, which indicates that this criterion is yet not strictly applicable. As observed with light microscopy, partial globozoospermia consists of a limited number of normal (oval) sperm cells next to aberrant forms (round-headed and dysmorphic sperm heads). The proportions differ from those found for both controls and patients with total globozoospermia. In TEM, we found that oval sperm cells in partial globozoospermia compared to normozoospermia often show sperm head construction defaults, such as space between the sperm head membranes and disarrangement of mitochondria next to partially formed acrosomes. The existence of partially formed acrosomes was confirmed by flow cytometry, which shows that some of the sperm cells of patients with partial globozoospermia contain acrosomal enzymes (proacrosin and acrosin), but lesser amounts than in the sperm cells of controls. The round-headed cells in partial globozoospermia were comparable to those in total globozoospermia for the examined features in TEM.

It is noteworthy that the significant differences between partial and total globozoospermia for all characteristics evaluated with TEM rather disappear in the analysis done according to head shape (oval or round). Although the unequal distribution of cells between the morphology groups is partly the cause, round sperm head morphology appears to be the most important discriminating factor. Round-headed sperm cells are accompanied by acrosome malformations and aberrant chromatin condensation. This implies that partial and total globozoospermia may have similar etiologic pathways leading to aberrant sperm head formation during spermiogenesis. The exact spermiogenetic defects in partial and total globozoospermia probably differ, but these entities may represent a variant phenotype of the same genetic factor(s). The phenotype of the mouse model *Csnk2A22/2*, at first wrongly classified as total globozoospermia, seems to share certain traits with partial globozoospermia (Xu et al,1999; Escalier et al, 2003). This gene is expressed in the brain and in the human testis, and it may play a role in globozoospermia, although additional physical features would then be expected. The combination of increased apoptosis in the early stages of spermatogenesis and the hampered maturation of the spermatids, as described by Escalier et al (2003), could form a starting point for speculation about the etiological pathways of partial globozoospermia. The Csnk2A2 protein appears to promote cell survival and opposes cell death in the apoptotic pathways by phosphorylating several proteins that induce caspase-mediated degradation during apoptosis (Escalier et al, 2003). We may speculate that partial globozo-

ospermia exists because the labeled defective spermatids do not follow apoptotic degradation as suggested by Sakkas et al (1999). Surprisingly, this effect described in oligozoospermia is not always observed in total globozoospermia (Dam et al, 2007a).

In other studies, total globozoospermia is always described as 100% round-headed and acrosomeless sperm round-headed sperm cells and acrosome malformations that exists separately from total globozoospermia. Partial globozoospermia contains oval sperm cells with distinctive malformations to the sperm head matrix compared to normozoospermia, but still normal sperm cells are found, which may be used in a clinical setting.

Acknowledgements

We owe our special thanks to Professor D. Escalier of the Andrology Department of Paris University, France, for kindly providing us with the antibody for proacrosin, which is not commercially available to date. We would also like to thank to Professor I. Fahmy of the Egyptian IVF-ET Center in Cairo, Egypt, for facilitating the inclusion of patients for this study and sending us the samples.

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Chapter 4

Sperm Integrity in partial globozoospermia

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Submitted

ABSTRACT

Partial globozoospermia is recently described as a teratozoospermia wherein at least 25 % of the sperm cells is round-headed and acrosomeless. Previously, we found indications for an aberrant spermiogenesis in partial globozoospermia. In this study we evaluated 10 partial globozoospermic patients and nine normozoospermic controls using computerised karyometric image analysis (CKIA) and DNA integrity by the immunofluorescence tests with TUNEL, CMA3 and KM-2 (tri-acetylated histone H4, indicative for early apoptosis or abnormalities in the spermiogenesis) and TH2B. All tests indicate that not only the total sperm population differs from normospermic controls as expected, but also the normal morphological sperm in PG differs in terms of DNA integrity. The significant difference in KM-2 staining intensity, observed in normal shaped cells, disappears when analyzed in morphologically abnormal cells in normozoospermia and partial globozoospermia. The testis-specific anti-histon 2B (TH2B) staining is more prominent in partial globozoospermia, although not significant different at sample level.

We conclude that early spermiogenesis is disturbed and that DNA damage is increased, although there is no significant difference in DNA damage compared to normozoospermia.

Key words: Sperm; partial globozoospermia; chromatin, DNA damage, CKIA

INTRODUCTION

Partial globozoospermia has been recently morphologically characterized as a subtype of teratozoospermia wherein at least 25 % of the ejaculated sperm cells is round-headed and a mean of 93% of the cells is acrosomeless, as determined by light microscopy [1]. Evaluation of partial globozoospermia by transmission electron microscopy (TEM) shows that round-headed cells present a rudimentary or a partial acrosome, which cannot be distinguished by conventional methods. Round-headed sperm cells in partial globozoospermia appear to be associated with poorly condensed chromatin, which implies an increased susceptibility to DNA damage. Previous studies in total globozoospermia show a variable chromatin structure and an increased DNA damage (see review[2]). Round-headed cells in partial globozoospermia and total globozoospermia also share an augmented percentage of other abnormal structural characteristics, such as space between nuclear membranes and displaced mitochondria [1]. In partial globozoospermia patients, similar structural abnormalities can be demonstrated in oval-headed ('normal-alike') sperm cells [1].

During spermiogenesis, sperm head elongation, acrosome formation and chromatin condensation take place, thereby transforming the spermatid into a mature spermatozoon [3, 4]. When acrosome formation is disturbed, the final steps in sperm maturation and morphology shaping are unbalanced and sperm cells develop as round-headed, usually acrosomeless or with partial/ rudimentary acrosome-like structure next to a poorly condensed chromatin, which suggests an association between these processes [1, 5, 6]. Interestingly, in partial globozoospermia the morphological 'normal' oval-headed as well as the round-headed shaped sperm show an increased percentage of structural abnormalities compared to normal or oval shaped sperm in normozoospermic controls, suggesting an aberrant maturation during spermiogenesis besides head shaping [1]. This finding has led us to further characterize sperm integrity and chromatin characteristics in partial globozoospermia in order to get a better insight of the probable risks for fertility treatment when using sperm from these patients.

The purpose of this study is to investigate sperm morphometric patterns using colorimetric techniques and computer evaluation (CKIA), and further evaluate sperm integrity using molecular techniques (immunofluorescence, IF) with TUNEL, CMA3, KM-2 and TH2B staining in order to obtain a more complete description of spermatozoa in partial globozoospermia.

MATERIALS AND METHODS

Patients

Patients were recruited at the Centre for Reproductive Medicine at Radboudumc, Nijmegen, the Netherlands. A total of 10 partial globozoospermia patients and nine normozoospermic controls were enrolled in this study. These partial globozoospermia and controls samples were also used in our previous study [1]. Patients' semen analyses are summarized in Table 1. Fresh sperm samples were analyzed following WHO criteria (motility and concentration) or strict criteria (morphology), and also stained for CKIA-analysis. The remnant of the sample was cryopreserved for further integrity analyses.

Because of the limited amount of material available, we could not perform all tests for all patients. For CMA3 and TUNEL, all samples were analyzed. For CKIA we used nine partial globozoospermia and all nine control samples. Immunofluorescence staining with anti-triacetylated H4 (KM-2) and anti-TH2B antibodies was possible in only seven partial globozoospermia and eight control samples.

CKIA

Feulgen stain and Computerized karyometric image analysis (CKIA)

Sperm head morphometric values were evaluated in detail using the computerized karyometric image analysis (CKIA). We previously adapted this technique, originally developed for urological cancer cells [7], to analyze human spermatozoa [8, 9]. The measurement specifications of the CKIA method have been extensively described previously [10, 11].

For the Feulgen stain, all chemicals were provided by Merck (Darmstadt, Germany). Air dried sperm samples were pre-fixed in a freshly prepared 100% Carbowax (2% polyethylenglycol, molecular weight 1500 in 50%)/ NaCl 0.9% solution (1/1) at 4ºC during 3 days. Subsequently the slides were fixed with 100% Carbowax at 4ºC for one day. The slides were immersed before staining in Böhm solution (10% formaldehyde [37%], 5% glacial acetic acid, 85% absolute ethanol). Staining was performed with Feulgen-Schiff stain (hydrolysis at room temperature in 5N HCl for 60 minutes and Schiff-reagent for 30 minutes). Cells were mounted in Permount (Fischer Scientific, Fairlawn, NJ, USA).

Feulgen stained sperm samples were evaluated digitally (CKIA), thereby yielding information on the 1) morphometry (size and shape of sperm cell), 2) densitometry (stain intensity of chromatin) and 3) chromatin texture (stain pattern distribution) of the cells analyzed. Only intact sperm cells were used.

1) Morphometric parameters: describes size and shape of the nucleus. Measures consist of *area* (number of pixels/μm^2), *fellicity* (minor axis/major axis, a measurement for head roundness), *ben* (nuclear roundness, arbitrary units,

	n	Vol. mL	Conc. x10⁶/mL	% Motil. (A+B)	% Morphology		% Acrosomeless	
		(range)	*(range)*	*(range)*	*(range)*		*(range)*	
					A/E	D-Q	A/E	D-Q
Ctrl	9	3.4 *(1.9–6.3)*	56 *(25–85)*	55 *(25–85)*	19 *(5–30)*	13 *(9–17)*	0 .	5 *(0–16)*
PG	10	2.9 *(0.8–5.0)*	21 *(4–50)*	22 *(10–35)*	2 *(0–5)*	1 *(0–4)*	58 *(30–82)*	53 *(25–83)*

Table 1 Semen characteristics of the patients and controls included in the study.

Ctrl= controls; PG= partial globozoospermia; Conc. = concentration; Motil. = motility (A+B) = progressive motility; ; % morphology = percentage of normal morphology according to strict criteria; A/E = aniline blue and eosin-Y staining; D-Q = Diff-Quik staining, % acrosomeless = percentage of sperm without acrosomes.

au), and nominal mean curvature (*nmac*: combines the head contour with smooth Freeman difference chain code of the cell perimeter, (au).

2) Densitometric parameters: related to DNA staining intensity. These parameters are the *OD* (optical density, au), *IOD* (integrated OD= area x OD (au)), and *varOD* (mean variation of the OD (au)).
3) Chromatin texture parameters: quantify stain distribution patterns. The *mean-his* (average OD along the maximal diameter (au)), the *SD-his* (standard deviation of the meanhis (au)), and the *CV-his* (coefficient of variation of the *SD-his*(au)).

Morphometric measurements with CKIA can evaluate each sperm in a complete and integral manner without observers' interpretation bias. In principle, at least 100 cells were evaluated per patient.

CMA3

Defective chromatin packaging was analyzed using the binding properties of chromomycine A3 (CMA3) (Sigma-Aldrich, Steinheim, Germany). CMA3 specifically binds to GC-rich sequences in the DNA in competition with protamines and it is associated with changes in protein composition, suggesting an incomplete protamination and condensation. Incomplete chromatin condensation is more susceptible for oxidative stress and can lead to increased DNA damage[12-15]. For CMA3 staining, the procedure described by Bianchi and colleagues [12] was used. A droplet of 5 μl of cell suspension was air-dried on a glass slide. Cells were fixed during 3 minutes in cold methanol/ acetic acid 3:1 and washed twice with phosphate buffered saline (PBS, Sigma, St Louis, MO, USA), pH 7.4. Each

slide was treated with 100 μl CMA3 solution (0.25 mg/ml in McIlvaine buffer, pH 7.0 containing 10 mmol $MgCl_2$) in the dark for 20 minutes at room temperature. Slides were washed three times (5 minutes) in PBS and mounted in mounting medium for fluorescence (Vectashield, Vector laboratories, Inc, Burlingame, CA, USA). Chromatin condensation was assessed by scoring CMA3 stained spermatozoa as positive or negative (positive cells are poorly condensed, therefore, stain intensity is high). Scoring was carried out using a Leitz DM RBE fluorescence microscope provided with a band pass filter (13, excitation BP 450-490 nm, emission LP 515). At least 200 cells were scored per sample by 2 trained technicians.

TUNEL

DNA damage was evaluated by TdT-UTP nick end-labelling (TUNEL) assay. This is a direct marker for DNA fragmentation, as it binds to the free 3'-OH ends in DNA strand breaks (DSB). The presence of DNA strand breaks is characteristic for late apoptosis [16].

The TUNEL assay (Cell Death Detection kit, Roche Biochemicals) was executed following the manufacturer's specifications with minor modifications [17]. Briefly, air-dried spermatozoa were fixated in 1% PFA in PBS for 10 min at room temperature and rinsed twice with PBS followed by permeabilization with 0.2% Triton X-100 in PBS, for 10 min. Nuclei were exposed to the TdT-labelled nucleotide mix for 60 min at 37°C. Slides were rinsed twice (5 min) in PBS and the sperm nuclei were counterstained with 4',6-diamidino-2-fenylindool (DAPI) (0.01 mg/l in PBS). Nuclei were mounted in 25 μl Vectashield. The total number of DAPI blue staining sperm nuclei per field was counted first. A minimum of 200, but mostly between 300 and 400, nuclei per fraction were scored by two independent observers.

Immunofluorescence staining with KM-2 (anti-H4) and anti-TH2B antibodies

KM-2 has been used as an early marker for somatic cell apoptosis, to differentiate DNA damage caused by oxidative stress post maturation from DNA damage by aberrant/incomplete chromatin remodeling [18, 19]. KM-2 is specific for H4 acetylated at K8, K12, and/or K16 [20].

Testicular histone 2B (TH2B), one of the testicular histone variants, is involved in the remodeling processes during spermatogenesis, capacitation and fertilization [21].

Cryopreserved samples from controls (n=8) and partial globozoospermic patients (n=7) were used. Because of a shortage of material we could not perform these test on all patients. From all samples, five slides were prepared following a decondensation protocol adapted from Ramos et al. [18]: cryopreserved sperm

was thawed for 5 min; washed once with Human Tubal fluid (HTF) (3000 rpm, 5 min) and resuspended in 50 µl HTF to be diluted in water 1:4. A 5 µl sperm suspension droplet was dried on a glass slide for about 10 min. 100 µl Dithiothreitol (DTT) solution was added and incubated in a humid incubation box for 10min. After removal of excess fluid, 100 µl decondensation mix (decondensation mix: 2.5 mM DTT/0.2% Triton X100/100 IU heparin/ml) was added and incubated in a humidified incubation box for 2- 3.5 min. The slides were fixed in a coplin jar with 4% PFA for 15 min, washed in PBS for 3x 5 min, rinsed in milliQ, air-dried and kept on 4 °C before use.

Immunofluorescence staining was performed for both controls and partial globozoospermic samples using the mouse monoclonal antibody KM-2 (1:4000) and rabbit polyclonal TH2B (Abcam (ab23913); dilution 1:5000. Alexa fluor 488 goat anti-mouse (1:500) and Alexa fluor 594 goat anti-rabbit (1:500) were used as secondary antibodies. The slides were washed 2 x 10min in PBS-0.1% Triton X100 at room temperature, then blocked for 1 hour at 37 °C with 200 µl blocking buffer per slide. After removal of excess fluid, 100µl of blocking buffer with 0.1%-Triton containing the first antibody was incubated at 37 °C for 20 min, and then incubated overnight at 4 °C. After continuous incubation at 37 °C for 20 min the next day, the slides were washed in PBS-Triton for 15 min. After removal of excess fluid, 200 µl blocking buffer was applied and incubated at 37 °C for 30 min. 100 µl per slide of the second antibody in blocking buffer was applied and incubated at 37 °C for 2 hours, then washed in PBS for 10 min. 200 µl DAPI (dilution 1:3000) was applied and incubated at room temperature for 10 min. The slides were then rinsed in PBS and covered with a coverslip after applying Vectashield and stored in the dark at 4 °C until further assessment.

Cells were localized and photographed with a Zeiss Axiophot2 Fluorescence microscope with Axiocam MRm CCD camera and Axiovision software. The DAPI, FITC and Texas red filters were used. The extent of decondensation and the fluorescence intensity were determined. Only well decondensed cells (+) were used for analysis. In order to semi-quantify the light and signal intensity, an ordinal representation was used (no signal (-); weak intensity (+-); strong intensity (+) and highly fluorescent nucleus (++). Representation of the score procedure in Figure 1. At least 50 cells were evaluated per sample.

Statistics

One way ANOVA was used for comparing the groups in CKIA, CMA3, TUNEL, KM-2 and anti-TH2B. The SPSS software pack 19.0 for Windows was used.

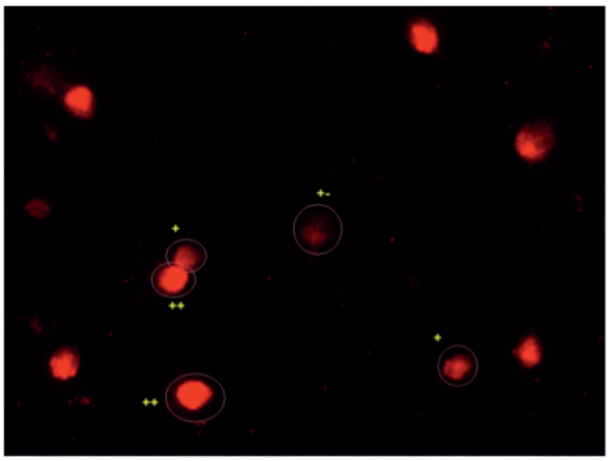


Figure 1 Representation of the score procedure.

In order to semi-quantify the light and signal intensity, an ordinal representation was used (no signal (-); weak intensity (+-); strong intensity (+) and highly fluorescent nucleus (++).

RESULTS

CKIA analysis

A total of 1032 and 1107 spermatozoa was analyzed in controls (range 89-153 cells/ sample) and partial globozoospermia patients (range 86-156 cells/ sample), respectively. The outcomes of the CKIA measurements are shown in Table 2A and 2B and Fig 2 and 3.

Figure 2 and Table 2A show a decreased percentage of normal cells (mean 4.3% vs.1.4%, p= 0.04) and a increased but not significant percentage of round-headed spermatozoa (mean 18.7% vs. 34.2%, p=0.08) in partial globozoospermia, as expected from the inclusion criteria used. Although this difference is not significant, it is in line with the former description of partial globozoospermia, wherein it is set as ≥25 % round headed cells [1].

The percentage of spermatozoa within the normal reference range for morphometry and densitometry parameters was significantly lower for partial globozoospermia samples compared to controls (ANOVA, $p < 0.001$) (Table 2A). Although individual parameters differ, the integrated normal chromatin texture measurements were comparable to controls. These data indicate normal sperm can be found in the ejaculates of partial globozoospermia males, however in low percentages compared to normospermic samples.

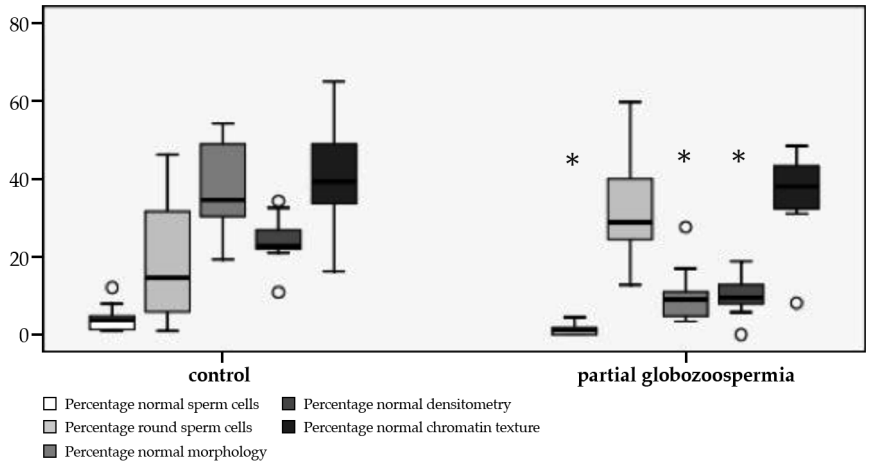


Figure 2 Morphometric parameters measured by CKIA in PG and controls.

Data for this figure are presented in Table 2A. (*) indicates significant difference.
- Percentages normal sperm cells: all integrated normal CKIA– values for sperm head normalcy
- Percentage round headed sperm is related to the shape of the head: fellicity values > 0.7 indicates a round headed sperm
- Percentage of cells in the categories morphometry, densitometry and chromatin texture: values within the normal ranges for each category.

	n	% Integrated normal CKIA morphology	% Round headed cells	% Normal Morphometry	% Normal Densitometry	% Normal Chromatine texture
Ctrl	1032	4.3	18.7	37.6	24.3	41.2
		0.9-12.10	*0.9-46.2*	*19.3-54.3*	*10.9-34.3*	*16.3-65.1*
PG	1107	1.4	34.2	10.6	9.8	35.6
		0-4.5	*12.8-59.8*	*3.4-27.6*	*0-18.9*	*0.9-48.5*
p		*0.04*	*0.06*	*<0.001*	*<0.001*	*0.37*

Table 2A Integrated morphometric evaluation.

In table 2A, the percentage of normal cells as evaluated by CKIA based on the normal reference values according to Ramos *et al*. (2004). The percentage integral normal CKIA-morphology is the percentage of cells considered normal per evaluated sample. This integral evaluation is based on 3 main parameters: morphometry, densitometry and chromatin texture. Specifically, the round headed sperm are given, which are those cases with a fellicity >0.7 um^2. P values ≤ 0.05 are considered statitstical different.

	N	Area (μm^2)	Fellicity (μm^2)	BEN (au)	Nmac (au)	OD (au)	IOD (au)	var-OD (au)	Mean-his (au)	SD-his (au)	CV-his (au)
Ref*		5.5 - 8.0	0.52-0.70	1100-1800	22-38	0.75-0.86	4.29-5.27	0.18-0.30	85-135	38-54	0.37-0.57
Ctrl	1032	6.39	0.61	1500	32.76	0.82	5.13	0.26	117.20	43.72	0.38
		4-10.7	*0.31-0.88*	*1000-3000*	*14-61*	*0.51-0.97*	*3.05-7.84*	*0.13-0.38*	*72-162*	*22-70*	*0.18-0.66*
PG	1107	5.10*	0.64	1444	35.02	0.90*	4.62	0.19	96.78	39.42	0.41
		1.7-19.3	*0.28-0.91*	*1000-3000*	*11-82*	*0.42-0.98*	*0.7-12.08*	*0.11-0.35*	*64-173*	*15-61*	*0.09-0.64*
p		**≤ 0.001**	**≤ 0.001**	**0.005**	**≤ 0.001**	**≤ 0.001**	**≤ 0.001**	**≤ 0.001**	**≤ 0.001**	**≤ 0.001**	**≤ 0.001**

Table 2B Analysis of the specific morphometric parameters evaluated with CKIA.

In Table 2B, the individual values of the measured paramenters are displayed and compared to the reference value . Statistical diffencences between from the reference values (*) are given. For all categories, Ctrl and PG differed (one way ANOVA).
Au= arbitrary units, .Ref= reference values; Ctrl: controls

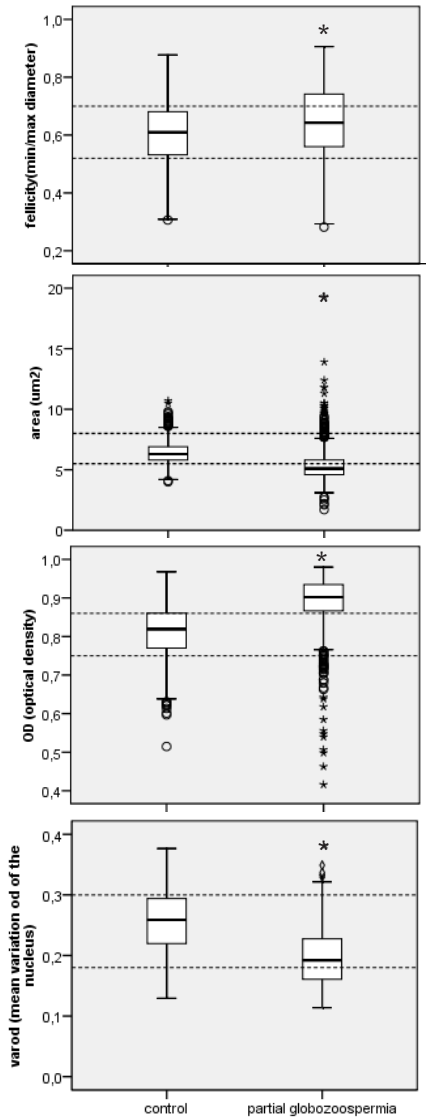
Morphometric parameters:
Area: nuclear area; Fell: felicity, elliptic factor (min/max diameter); BEN: bending energy
(difference between highest and lowest value in the smooth Freeman difference chain code (SFDC))
Nmac: nominal mean curvature derived from SFDC
Densitometric parameters:
OD: optical density (mean optical density, stainability of the cell); IOD: integrated optical density (area x OD);
var-OD: mean variation of the OD of the nucleus;.
Chromatin texture parameters:
Mean-his: mean grey value of the line of the maximal diameter; SD-his: mean standard deviation of the grey values
of the line of the maximal diameter; CV-his: mean coefficient of variation of sdhis.

In table 2B, the individual morphometric parameters are given. The mean values of the control samples are displayed, which are comparable to earlier defined normal reference ranges [10]. Although all individual CKIA variables were significantly different between controls and partial globozoospermia, for the total sample, the mean values for roundness (fell); BEN; nmac; IOD; varOD; meanhis, sdhis and cvhis remained within the defined reference ranges for both groups, again indicating that sperm still do meet the values defined for normality. We should take into account that the mean values reflect the average of a population and are therefore not representative for single cells. The smaller area and higher OD (staining) is indicative for sperm with a small nucleus and poorly condensed chromatin, which allows a better binding of the Feulgen stain to DNA, increasing the OD. Figure 3 (left side) displays the differences in parameters (felicity, area, OD en varOD) between partial globozoospermia and controls. In the right side of figure 3 the differences in these paramenters has been given for the 'normal/oval shaped sperm' vs. the 'round headed sperm' found in partial globozoospermia and controls samples. Interestingly, normal shaped sperm differs between partial globozoospermia and control samples.

Molecular evaluation of sperm integrity using immunofluorescence

The results for CMA3, TUNEL, anti-Th2B and KM-2 are summarized in Fig 4.

For CMA3 a total of 2347 sperm (range 179-343 in nine controls) and 2596 sperm (range 230-309 in 10 partial globozoospermia patients) were scored. No statistical difference was found for CMA3 positive sperm in partial globozoospermia (mean 18.6%) and controls (mean 14.5%), respectively. For TUNEL, we scored 5786 (range 452-831 in nine controls) and 6210 spermatozoa (range 423-812 in 10 partial globozoospermia patients) respectively. A tendency for a higher percentage of TUNEL positive cells was observed in partial globozoospermia (mean 29.7 % vs. 19.8 % in controls), but this difference was not statistical significant (p= 0.077). Although a disturbed protamination and abnormal compactation (CKIA, CMA3 +) and higher levels of DNA damage (TUNEL) are observed in partial globozoospermia, at sample level these values do not statistically differ from controls samples For immunofluorescence staining, decondensation of the sperm head is crucial in order to be sure that the antibodies can reach the whole nucleus and assure the binding to all histones present. Still after decondensation, morphology can be evaluated as the shape is retained but the volume is increased. Only proper decondensed sperm was scored for KM-2 and TH2B staining (Table 3). TH2B staining is more prominent in partial globozoospermia, indicating a higher retention of histones, but did not reach significant difference. For H4 acetylated at K8, K12, and/or K16 (stained with KM-2) 444 sperm (eight controls) and 385 sperm (seven globozoospermia patients) were


fellicity(min/max diameter)
1,0
0,8
0,6
0,4
0,2
area (um2)
20
15
10
5
0
OD (optical density)
1,0
0,9
0,8
0,7
0,6
0,5
0,4
varod (mean variation od of the nucleus)
0,4
0,3
0,2
0,1
0,0
control
partial globozoospermia

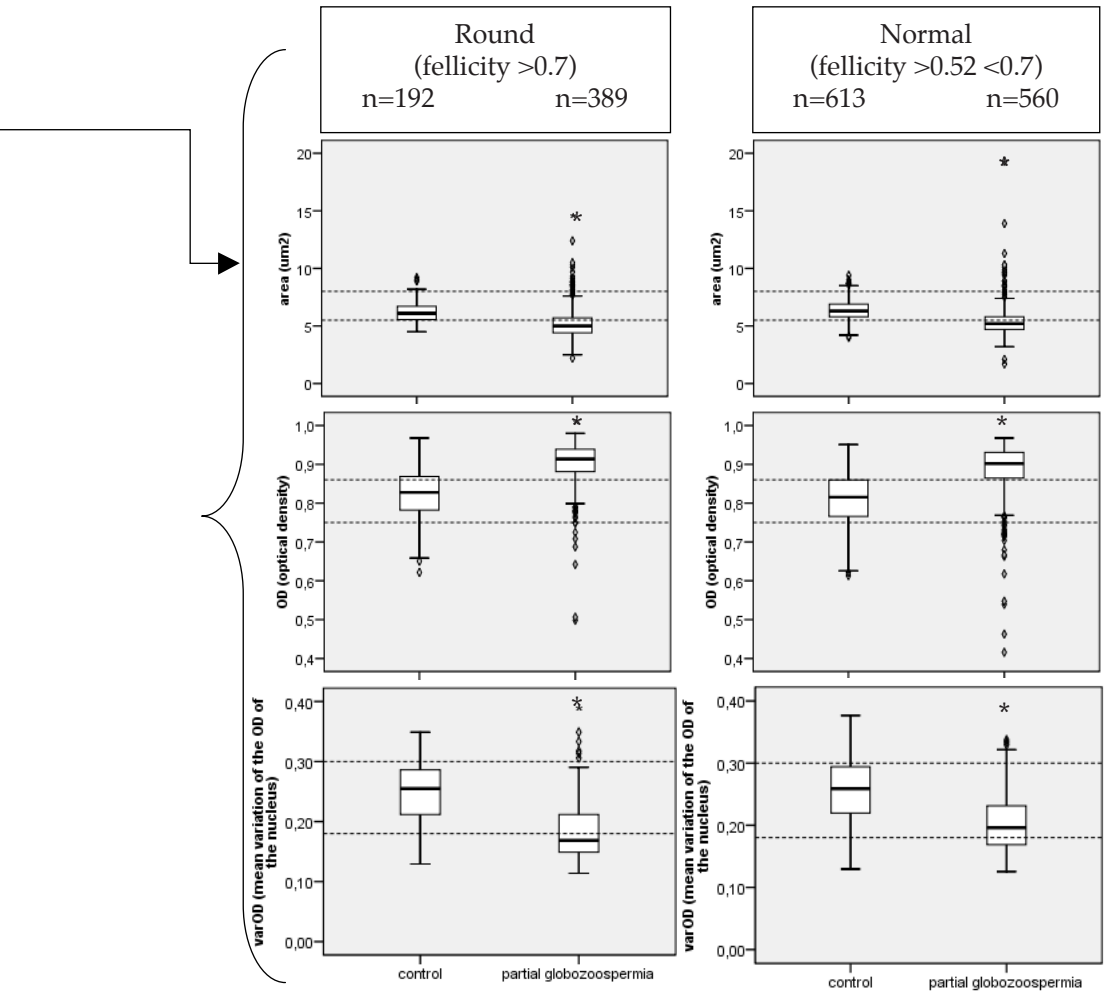


Figure 3 Representation of individual CKIA measurements of PG and controls for all sample (left) or round/normal shaped sperm (right).

PG (partial globozoospermia). *= significantly different form controls
Left, from top to bottom: mean values for fellicity, area, OD and var-OD for the total group of controls and PG respectively. **Middle**: mean values for area, OD and var-OD for the round-headed sperm cells (fellicity ≥0.7um2) in controls and PG, respectively. **Right**: mean values for area, OD and var-OD for the normal headed sperm cells (fellicity ≥0.52 and ≤0.7 um2). The reference lines indicate the normal ranges.

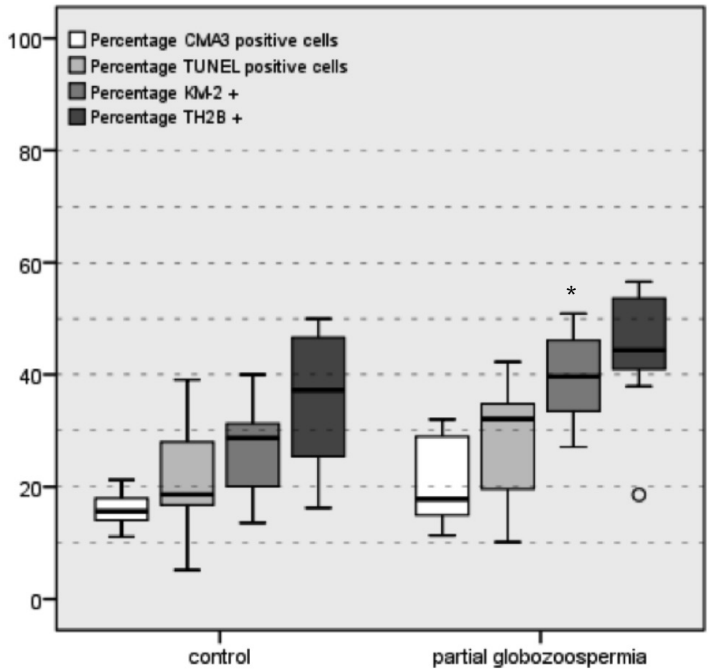


Figure 4 Chromatin integrity in PG.

Boxplot representation of the spermatozoa stained with CMA3, TUNEL, KM-2 and TH2B, indicating the population of damaged cells per studied group (control or partial globozoospermia). The percentage of KM-2 (+) cells is significantly different between controls and PG.

scored. KM-2 was found to be increased in partial globozoospermia (p= 0.014). When all sperm independently of the morphology were included, KM-2 staining was significantly different between partial globozoospermia and controls. However, when sperm morphology ('normal or abnormal') was taken into account, differences for KM-2 staining emerged for the 'normal-shaped' group: KM-2 (-) 18 % vs. controls 9%; and KM-2 (+) 40 % vs. 27% respectively (p=0.007). In the 'abnormal-shaped' group the statistical difference disappeared and similar amount of high positivity KM-2 (+/++) was observed (67% vs. controls 62%) respectively. In summary, chromatin integrity in spermatozoa from partial globozoospermia shows signs of a disturbed deacetylation at the start of spermiogenesis.

Morphology	Group	n	% KM-2				p	% TH2B				p
			-	+/-	+	++		-	+/-	+	++	
Total sample	Ctrl	444	24	32	27	17	0.014	9	32	36	22	ns
	PG	385	10*	29	40*	21		1	28	44	27	
Normal	Ctrl	195	18	37	27	17	0.007	1	33	40	26	ns
	PG	195	9*	32	40*	19		0	30	44	26	
Abnormal	Ctrl	47	10	28	30	32	ns	0	19	40	41	ns
	PG	149	10	22	43	25		1	24	44	31	

Table 3 Sperm integrity in morphological normal/abnormal sperm by KM-2 and TH2B markers.

Positivity of spermatozoa depending on the normal/abnormal shape of the sperm head after immunofluorescence of KM-2 and anti-TH2B is shown. Unselected well decondensed spermatozoa (total sample) showed significant differences in positivity after KM-2 (+) staining between controls and partial globozoospermia. Normal shaped sperm stained more positive (+) in the partial globozoospermia compared to the control group ($p<0.007$, at the expense of KM-2 negative (-) cells (one way ANOVA). Abnormal shaped sperm showed a higher rate of high intensity stained sperm with both KM-2 and TH2B markers, no difference between partial globozoospermia and controls were observed.
Ctrl= controls; PG=partial globozoospermia.

DISCUSSION

Partial globozoospermia is an aberrant kind of teratozoospermia, with structural abnormalities that occur during spermiogenesis. One of the main changes in this maturation phase is chromatin condensation, protamin eviction, formation of the acrosome and final sperm shaping.

As expected from the inclusion criteria, the evaluation by CKIA corroborates the presence of more round-headed cells ($p=0.06$) and significantly less normally shaped sperm cells ($p=0.04$) in partial globozoospermia patients. Mainly the sperm head area and optical density (OD) differed. When analysed in round-headed cells, also the median value of varOD fell outside the reference values. Combined chromatin densitometry indicated a significantly less well condensed chromatin in partial globozoospermia ($p<0.001$). On the other hand, still normal cells within partial globozoospermia samples (1.4%). Integral normal CKIA morphology vs. controls 4.3% $p= 0.04$) can be found, which can explain the successful clinical outcome when treating these patients with ICSI [22]. KM-2 staining shows signs of a disturbed deacetylation at the start of spermiogenesis ($p= 0.014$), also in normal shaped cells ($p= 0.007$). However, markers of disturbed chromatin condensation (CMA3), disturbed protamination (TH2B) or DNA damage (TUNEL) did not reach significance, although a tendency to an augmented level of (potential) damage was demonstrated.

The study population used here is the same as in our previous study [1], which confirms the presence of other molecular/structural anomalies which cannot be found using conventional techniques.

In this study, using the morphometrical data obtained by CKIA, we found clearly less condensed chromatin in partial globozoospermia, a difference that increases after analyzing the samples separately for OD and varOD in normal and round-headed cells respectively (Figure 2). The latter can be explained by the acrosome malformation and high stainability of the round-headed sperm. Notably, the smaller area of the spermatozoa in partial globozoospermia compared to controls is a distinct, inexplicable feature that was not observed before and seems to be in contradiction to the poor condensation of the chromatin in these samples.

It is unclear why in this typical type of teratozoospermia the CMA3 levels were lower than in the OAT samples described before by others [13, 18, 23]. Interestingly, this observation is also valid for the measurement of DNA fragmentation by TUNEL: a higher TUNEL staining intensity was observed in partial globozoospermia, but not significantly different from controls at sample level. To back up these observations by other chromatin markers, we applied IF with monoclonal antibodies for a testicular histone variant (TH2B, as a marker

of incomplete protamination and remodeling) and a specific histone modification that is a tri-acetylation pattern on H4, recognized by the antibody KM-2.

TH2B is a histone variant that is present in early spermatogenesis when it replaces the somatic H2B in the spermatogonia, but is most expressed in round spermatids after meiosis [24]. In contradiction to TH2A, which is not expressed beyond meiosis, TH2B remains throughout spermiogenesis and is not entirely replaced after protamination. Observations of this process show that replacement of histone by protamines follows a specific pattern starting from acrosome to the caudal pole of the spermatid [24]. In normal ejaculated sperm samples, TH2B is present in a subfraction of the sperm samples, approximately in about 20-40% of the sperm cells [18, 19, 25]. It is remarkable that in normal sperm samples this histone variant is almost always present in low levels, suggesting a role in regulating genes that take part in spermiogenesis itself [26], but when present in high levels (observed with IF; + and ++ fractions), incomplete spermiogenesis should be suspected. Therefore, low levels of anti-TH2B staining (+/-) in sperm can be considered to be a positive marker for sperm maturation as suggested [18]. In our current study we found higher percentage of anti-TH2B staining (++) in abnormal cells for both controls and in partial globozoospermia (41% vs. 31%) compared with the normal (oval) sperms (26% vs. 26% respectively, see table 3). This is however different from literature in which percentages of 1% [18] or up to 23% in normozoospermia and 46 % in case of OAT [19] has been described. One explanation for such variation in results may relay in the type of antibody used: we used rabbit polyclonal TH2B antibody instead of tyrosine hydroxylase antibody [19, 24, 27]. The rabbit polyclonal antibody used in this study recognizes the amino acids ATISK**KGF**KK instead of peptide KGF in case of the anti-tyrosine hydroxylase antibody, making the first one more specific for this testicular histone variant. We do observe the same trend in partial globozoospermia samples as in the OAT population that was described previously[19]. This might indicate that the presence of TH2B at low levels could be a positive predictor for normal condensation, however, too high levels of TH2B may indicate an incomplete protamination, therefore, abnormal chromatin condensation inducing poor fertilization. In our study the anti-TH2B stain intensity in poor decondensed sperm is stronger in partial globozoospermia, indicating that the accessibility to the antibody is not impaired but an abnormal high TH2B content is related to less well-organized chromatin.

The histone modification that recognizes H4 acetylated at lysine 8, 12 and 16 (H4acK8,12,16) is called KM-2 in this and other studies of our group. Histone 4 acetylation takes place before meiosis, is deacetylated during meiosis, but is reacetylated, probably just like H3, in the elongating spermatid and it is known as a prerequisite for the histone-to-protamine exchange [28]. Hyperacetylated

H4 is normally detected in all spermatids at the start of elongation in normal spermatogenesis, but in only 75-90 % of the spermatids in case of OAT, probably due to premature or decreased acetylation of H4 leading to a disturbed protamination or even a maturation arrest [29]. Its presence in ejaculated sperm cells may indicate an early sign of apoptosis or abnormal chromatin maturation [18, 19]. In this study we see a significantly increased percentage of KM-2 (+) cells in partial globozoospermia compared to controls. This difference is even more present in normally shaped sperm cells, indicating that chromatin condensation may also be disturbed in apparently normal cells in partial globozoospermia. This finding is a precautionary conclusion because of the low numbers analysed. The difference disappears when the abnormal cells in both study groups are compared (both are elevated), which seems proof of the theory that KM-2 positive cells implicate a disturbed spermiogenesis at the level of sperm head shaping. Here, the percentage of KM-2 positive cells is higher than in previous studies from our group [18, 19].

From a biologic point of view, partial globozoospermia emerges to be a remarkable sperm phenotype in order to better understand spermiogenesis. In 1971 Schirren et al. [30] described the etiology of (total) globozoospermia as the formation of the acrosome in the cytoplasm, seperated of the sperm head. The acrosome was then supposed to be lost in the cytoplasmic droplet, resulting in a round-headed sperm head. Nowadays, it appears that the elongation of the sperm head, the formation of the acrosome and chromatin condensation are processes that are not causally related but occur simultaneously, communicating amongst each other during the process [4]. This latter study shows that all three processes: sperm head elongation, protamination and acrosome formation start at the top of the sperm head and go down to the tail at a strict coordinated sequential process. In our earlier study on partial globozoospermia, we noted that chromatin condensation was equally disturbed in round-headed cells of controls, partial globozoospermia or total globozoospermia, indicating a common pathway disturbance. Also, we observed that even oval shaped sperm cells in partial globozoospermia displayed structural anomalies regarding the head matrix [1], suggesting that the maturation processes during spermiogenesis of these men are affected independently of the final shape of the sperm. One can speculate that the chromatin aberrations might arise from a mild or more severe communication disorder during spermiogenesis, or a shortcoming in repair mechanisms. Unclear is whether this “miscommunication” is due to a genetic origin or to a temporal effect such as disease, drugs, medication or another environmental effect. Total globozoospermia is observed at all sperm cells of one individual; however, the percentage of round-headed sperm in partial globozoospermia can vary between semen analyses in one individual in time.

Notably, in this study we observed that also normal sperm cells are produced that can be used in case of ICSI [22].

In conclusion, there are several indications that early processes during spermiogenesis are disturbed and that DNA damage may be increased in partial globozoospermia, although these defects do not affect all sperm in the sample. From the clinical point of view, it is reassuring that these patients performed so well in ART (assisted reproductive technology) programs, with good fertilisation and pregnancy rates and without increased miscarriage rates [22].

Acknowledgements

We would like to thank Leonie van den Hoven and Hannie Robben for collecting and cryopreserving sperm samples at the IVF-lab of the RUNMC. A special thanks for Dr. Peter de Boer for his enthusiasm and advice.

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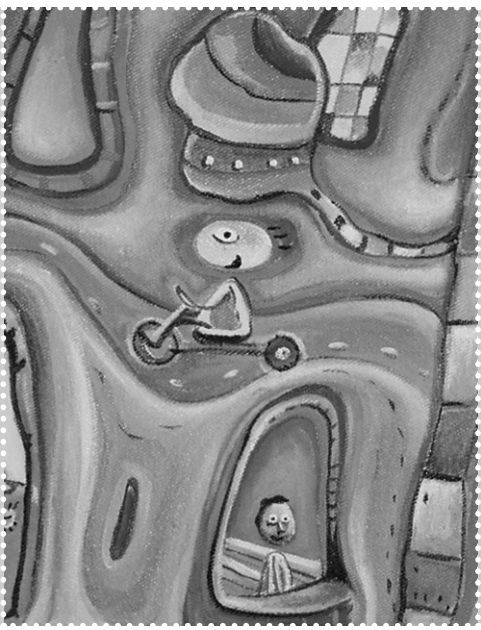
Chapter 5

Intracytoplasmic sperm injection in partial globozoospermia



Dam A.H.D.M., Pijnenburg A.J.E., Hendriks J.C.M., Westphal J.R.,
Ramos L., Kremer J.A.M.

Fertil Steril, 2012. 97(1): p. 60-66.

ABSTRACT

Objective: To compare the outcome of intracytoplasmic sperm injection (ICSI) in patient couples where the male partner has partial globozoospermia with the outcome in a general ICSI population.
Design: Case-control study.
Setting: Center for Reproductive Medicine, Radboud University Nijmegen Medical Center, the Netherlands, a tertiary referral center.
Patient(s): Between 1997 and 2005, 42 couples were identified in which the male presented with partial globozoospermia; 27 couples treated with ICSI were matched with 263 control couples from a general ICSI population regarding female age and year of first ICSI cycle.
Intervention(s): One ICSI treatment (1–10 ICSI cycles).
Main Outcome Measure(s): Live birth rate after one ICSI treatment (1–10 ICSI cycles).
Result(s): In the partial globozoospermia group, the live birth rate was 66.7% compared with 50.0% in the control group. In partial globozoospermia, three out of 21 pregnancies ended in a miscarriage, one major birth defect occurred, and one pregnancy ended in a neonatal death due to sepsis in a premature child, compared with four stillborn in the control group.
Conclusion(s): ICSI is an effective treatment in couples that failed to conceive spontaneously within 1 year combined with male infertility due to partial globozoospermia. The fertilization rates and the live birth rates in this specific group did not differ from those of the general ICSI population.

Key Words: Partial globozoospermia, sperm morphology disorder, ICSI

INTRODUCTION

Partial globozoospermia is a recently described type of teratozoospermia that is characterized by an increased amount of round-headed sperm cells and typical acrosome defects. Remarkably, the oval spermatozoa derived from PG patients display an increased incidence of differentiation defects as well. Although the round-headed cells that are present in partial and total globozoospermia appear alike regarding additional structural defects, partial and complete globozoospermia may represent different disorders (1).

The first pregnancy using round- headed spermatozoa derived from a patient suffering from total globozoospermia was achieved in 1994 with the use of intracytoplasmic sperm injection (ICSI) (2). In subsequent cases, fertilization rates were poor in 50% (3). The poor fertilization rates with ICSI may be due to deficient oocyte activation by these spermatozoa (4). Oocyte activation with calcium ionophores or electric activation has been applied in some studies and resulted in better fertilization rates in most cases (3, 5–11). To our knowledge, no augmented risk on congenital abnormalities has been reported in such pregnancies.

In partial globozoospermia, the ejaculate contains not only round- headed spermatozoa, but also a certain percentage of normal sperm cells (1). A spontaneous conception is therefore possible in theory, although we expect that these patients may have impaired fertility and a need for medical assistance in conceiving a child.

To date, there are no studies on the need and the efficacy of assisted reproductive techniques (ART) in this specific group of patients. Because we found defects in spermatozoa that appeared normally shaped in an earlier study (1), this disorder may lead to an unknown cause for poor fertilization, as in total globozoospermia. The aim of the present study was therefore to assess the success rate as well as the possible adverse risks of ART for patients suffering from this rare kind of teratozoospermia.

MATERIALS AND METHODS

Fertility Treatment

In our center, patients are offered fertility treatment according to the guidelines of the Dutch Society of Obstetrics and Gynecology. This means that couples are evaluated by the family doctor after a 1-year existence of a fertility disorder. If necessary, they are referred to a hospital. In case of male subfertility, the VCM (volume concentration motility count, without processing) is calculated to determine the severity of the male infertility to provide the best treatment. A VCM of $>3\ 10^6$ sperm cells/ mL is considered to be mild male subfertility, for which expectant

management is pursued. A VCM of 1–3 10^6/mL, is considered to be moderate, for which treatment is indicated in the form of intrauterine insemination (IUI, homologous) or in vitro fertilization (IVF). In case of a severe male subfertility (VCM <1 10^6/mL), IVF or ICSI is offered; in case of VCM <0.5 10^6, ICSI is offered as the only solution. In daily practice, ICSI is offered when total fertilization failure occurs in IVF. Patients are counseled and treated according to this guideline (17).

Partial Globozoospermia Group

PG patients were collected at the Radboud University Nijmegen Medical Center of Reproduction between 1997 and 2005. In total, 42 couples were identified with the male partner presenting with partial globozoospermia (>25% round-headed or acrosomeless sperm cells in the ejaculate) (1). All of the couples suffered from a primary fertility disorder. These couples were enrolled in our assisted fertility program (Fig. 1). Ten couples eventually did not receive any treatment. Three of these couples (patients 3, 5, and 6) conceived spontaneously with a time to pregnancy between 1 and 3 years. Two patients (patients 1 and 8) decided not to make use of assisted reproductive technologies (ART), and finally, five couples were referred elsewhere for treatment (patients 2, 4, 7, 9, and 10). The remaining 32 couples received ART IUI, IVF, or ICSI at our center. A flowchart of the patient treatment and outcomes is shown in Figure 1. Two out of the three couples undergoing IUI (patients 11 and 12) conceived after six and four cycles, respectively. The third couple ceased treatment after two cycles (patient 13). IVF was performed in eight couples: Total fertilization failure occurred in five couples (patients 15, 17–19, and 21), and poor fertilization (27% and 8%, respectively) in two couples (patients 16 and 20). These seven couples were referred for ICSI. The last couple ceased treatment after two cycles of IVF (patient 14). From the 28 couples that started ICSI treatment (patients 22–42), one couple was excluded for further analysis because no ovum pick-up (OPU) could be performed (patient 22).

We compared the outcome of the 27 PG patient couples who underwent ICSI with the outcome in a general ICSI population with no known partial globozoospermia.

Control Subjects

Each PG patient couple that was treated with ICSI was intended to be matched with ten control couples. For seven control couples, sufficient data were not available, and they were therefore excluded from further analysis, resulting in a total of 263 control couples and 27 PG couples for comparative analysis. Matching was performed in a random fashion from the cohort of couples receiving ICSI in our center.

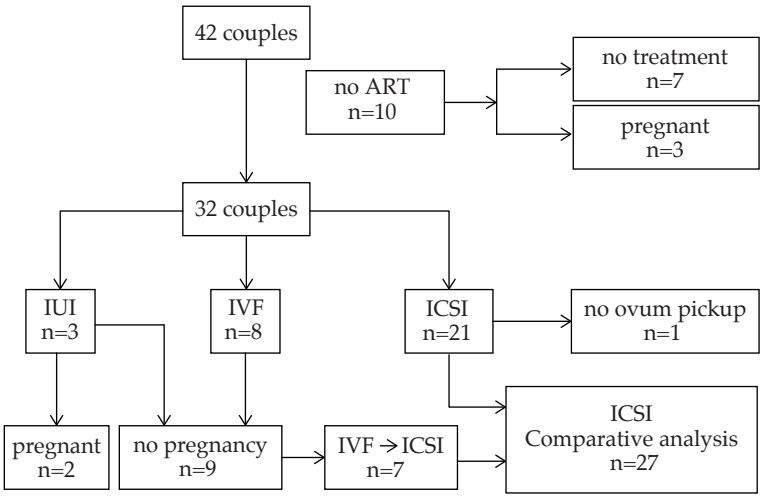


Figure 1 Flow chart of number of patient couples and treatment offered in partial globozoospermia.

ART = assisted reproductive technologies; ICSI = intracytoplasmic sperm injection; IUI = intrauterine insemination; IVF = in vitro fertilization.

Matching criteria were female age at the time of the first OPU and year of the first ICSI cycle. These matching criteria were chosen for the well-known influence of female age on ART outcome and to compensate for slight changes in protocols that may have occurred over the years, respectively. All control couples suffered from a primary fertility disorder, they were selected for treatment according to the guideline, and none of the male partners suffered from (partial) globozoospermia.

ICSI Treatment

Only the first ICSI treatment was included for analysis. Note that a single treatment may contain several ICSI cycles (1–10 cycles in this study).

Ovarian stimulation was performed by administration of GnRH agonist starting on day 21 of the previous cycle, followed by a stimulation phase with recombinant FSH. OPU was planned when R13 follicles with a diameter of >18 mm were observed by ultrasound examination, and OPU was performed 36 hours after hCG injection. ICSI was performed basically as described previously by Palermo et al. (12). Fertilization rates were scored 18–22 hours after the ICSI procedure. On day 3 after OPU, the embryos were scored regarding number of

blastomeres and percentage of fragmentation. Embryos were classified as A, B or C, with A embryos having the highest chance of implantation and C embryos the lowest.

The best embryos were selected for single-embryo transfer (SET) or double-embryo transfer (DET). Embryo cryopreservation was performed on surplus class A embryos. A pregnancy test was performed on day 18 after OPU, and an ultrasound was made 3–5 weeks after the embryo transfer. One or more intrauterine gestational sacs and fetal heartbeat were considered to indicate a pregnancy. A miscarriage was considered as such before 16 weeks of gestation. Pregnancy and neonatal outcome were collected by patient questionnaires (13).

DATA COLLECTION

The live birth rate was set as the primary outcome measure, and clinical pregnancies, miscarriages, stillborns, and twins were secondary outcome measures. Medical records of all patient and control couples were reviewed and the following data collected: female date of birth, date of the first and last OPU, start date of fertility disorder, number of ICSI cycles (including cryo-embryo tranfer), total number of obtained oocytes, fertilization rate, total number of transferred embryos (cryo-embryos included), embryo quality (A [excellent], B [good-regular], and C [poor]); the presence of cryo-embryo transfer as a secondary marker for embryo quality, and outcome of the treatment: live birth, miscarriages, twins, and stillborns. Detailed information on the newborns in the PG group was also collected. The following figures were calculated per treatment: percentage of live births, percentage of miscarriages, number of ICSI cycles, presence of cryocycles, and percentages of quality A, B, or C embryos (Table 1). Female age and duration of fertility disorder (years) were calculated by female date of birth and start date of fertility disorder compared with the date of the first OPU. Duration of treatment (years) was calculated by the date of the first OPU compared with the date of the last OPU. The following figures were calculated per cycle: number of obtained oocytes, fertilization rate, and number of transferred embryos.

Statistical Methods

This study was designed to study differences between PG patient couples and matched control couples. Each patient couple was intended to be matched with ten control couples. A linear mixed model was used to test the difference between the patient group and the control group that accounts for the matched design (multilevel model). The dependent variable was duration of the fertility

disorder, duration of treatment, fertilization rate, quality of transferred embryos, and number of obtained oocytes, respectively. The independent variable was group (PG patient, control), and the match number was treated as a random effect.

A conditional logistic regression model was used in case of dichotomous outcomes. The dependent variable was number of cycles (dichotomized as <2 or >2), number of transferred embryos (dichotomized as <2 or >2), presence of cryopreservation, pregnancy, and live birth, respectively. Again, the independent class variable was group (PG patient, control), and the match number was the identifier of the strata (levels).

The Fisher exact test was used to test the difference between groups of percentage of miscarriages, singletons, twins, and stillborns as a proportion of the pregnancies for statistical significance. A t-test was performed to compare means of the semen parameters in the pregnant and nonpregnant couples. Note that a matched analysis is not applicable in these cases. A P value of $< .05$ was considered to be statistically significant. The statistical analysis was performed using SAS 9.2 for Windows and SPSS 16.0 for Windows.

RESULTS

Between 1997 and 2005, 42 subfertile couples in which the male suffered from partial globozoospermia were identified (Fig. 1). Of these 42 couples, 27 underwent ICSI. The first ICSI treatments of PG patient couples were included for analysis and were matched with 263 first ICSI treatments in the control group.

Table 2 presents the patient semen parameters with the fertility treatment and outcome. In the patient couples that conceived spontaneously or by IUI, no more than a mild oligozoospermia or asthenozoospermia was found in the semen analysis, rather than an obvious teratozoospermia. Although these values are not that different from the semen analysis of the couples that initially underwent IVF and were found to have a poor fertilization rate, they are different from the values of the patients that underwent ICSI as initial treatment (concentration: $P < .001$; motility: $P = .012$). The mean semen parameters, including the percentage of acrosomeless sperm cells, were not significantly different between the pregnant and non-pregnant ICSI couples.

Table 1 presents the characteristics of the patient and control groups. The patient and control groups were similar regarding female age because this was a matching criterion. Also, duration of the fertility disorder, duration of the treatment, and number of ICSI cycles were similar in the two groups. No significant differences were found for number of oocytes obtained per cycle,

Pat.	Treat ment	n	Volume (mL)		Concentration (*10⁶/mL)		A+B Motility (%)		Morphology (%)		Acrosomeless (%)		Incl.	Preg. (Y/N)
			med.	*range*	med.	*range*	med.	*range*	med.	*range*	med.	*range*		
1	None	1	2.9	-	35		30	-	18		26	-	N	N
2	None	5	0.9	*(0.7-1.7)*	1.5	*(1-7)*	10	*(5-25)*	0	*(0-0)*	75	*(75-99)*	N	N
3	None	1	2.1	-	65	-	25	-	17	-	54	-	N	Y
4	None	1	3.6	-	35	-	55	-	9	-	40	-	N	N
5	None	1	2.0	-	15	-	35	-	0	-	85	-	N	Y
6	None	2	2.4	*(2.4-2.4)*	65	*(30-100)*	60	*(55-65)*	1	*(1-1)*	28	*(15-40)*	N	Y
7	None	1	8.3	-	0.5	-	1	-	0	-	99	-	N	N
8	None	1	4.8	-	1.5	-	15	-	0	-	60	-	N	N
9	None	1	1.8	-	50	-	35	-	0	-	80	-	N	N
10	None	6	3.3	*(1.5-6.2)*	0.3	*(0.1-2)*	7	*(4-15)*	0	-	65	*(50-84)*	N	N
11	IUI	3	3.5	*(3.3-4.8)*	15	*(15-20)*	22	*(9-39)*	1	*(0-1)*	60	-	N	Y
12	IUI	4	2.9	*(1.6-3.3)*	9.5	*(5-40)*	27	*(10-55)*	1	*(1-2)*	50	-	N	Y
13	IUI	2	5.5	*(5.2-5.9)*	13.5	*(10-17)*	23	*(20-25)*	2	*(1-3)*	65	*(60-70)*	N	N
14	IVF	1	4.1	-	10	-	30	-	1	-	66	-	N	N
15	ICSI[1]	1	2.0	-	10	-	10	-	1	-	80	-	N	N
16	ICSI[3]	5	2.0	*(1.4-3.6)*	50	*(11-95)*	35	*(20-50)*	2	*(1-6)*	73	*(60-77)*	Y	Y
17	ICSI[2]	4	4.4	*(3.5-4.7)*	15.5	*(4-20)*	40	*(15-55)*	0	*(0-1)*	64	*(60-73)*	Y	Y
18	ICSI[3]	3	1.8	*(0.5-2.0)*	4	*(2-30)*	35	*(10-45)*	2	*(1-3)*	50	-	Y	Y
19	ICSI[3]	3	4.9	*(3.4-5.1)*	13.3	*(5-20)*	35	*(20-45)*	1	*(1-3)*	50	*(40-55)*	Y	Y
20	ICSI[3]	6	5.2	*(4.7-6.0)*	45	*(16-50)*	43	*(40-65)*	1	*(1-2)*	60	*(40-60)*	Y	Y
21	ICSI[2]	3	5.3	*(3.7-8.2)*	43	*(30-85)*	35	*(20-35)*	4	*(4-4)*	41	*(32-50)*	Y	Y
22	ICSI[3]	3	5.0	*(4.1-5.4)*	2	*(1.2-4)*	55	*(20-55)*	1	-	60	-	Y	Y

23	ICSI	3	2.3	*(1.2-3.0)*	6	*(2.6-11)*	15	*(10-30)*	1	-	92	*(88-96)*	Y	Y
24	ICSI	4	*11.5	*(8.5-12)*	7.2	*(4-15)*	23	*(10-30)*	6	*(5-7)*	30	-	Y	Y
25	ICSI	4	2.3	*(1.9-3.0)*	11	*(5-37)*	1	*(1-5)*	15	*(14-24)*	86	-	Y	N
26	ICSI	5	*7.1	*(1.9-7.5)*	3.2	*(2.0-4.0)*	15	*(5-20)*	2	*(0-5)*	58	*(10-73)*	Y	Y
27	ICSI	1	2.2	-	1.5	-	10	-	7	-	?#	-	Y	Y
28	ICSI	3	2.0	*(1.9-2.9)*	12	*(4.5-15)*	50	*(35-60)*	0	-	50	-	Y	N
29	ICSI	3	4.8	*(3.3-4.9)*	25	*(17-30)*	10	*(10-20)*	0	-	75	*(73-76)*	Y	N
30	ICSI	3	4.5	*(3.9-4.9)*	1.1	*(1-2.8)*	10	*(10-40)*	2	*(0-5)*	83	-	Y	Y
31	ICSI	2	4.2	*(3.5-4.8)*	3.3	*(2.6-4)*	15	-	1	*(0-2)*	70	-	Y	N
32	ICSI	5	2.1	*(1.7-3.6)*	6	*(2.6-18)*	15	*(2-35)*	3	*(0-5)*	60	-	Y	Y
33	ICSI	3	3.5	*(2.8-4.0)*	1.1	*(0.9-1.5)*	5	*(1-6)*	0	-	65	-	Y	Y
34	ICSI	4	*6.9	*(4.6-8.7)*	12.5	*(4-25)*	11	*(10-20)*	1	*(0-6)*	60	-	Y	N
35	ICSI	3	1.2	*(1.1-2.2)*	11	*(4-15)*	15	*(10-45)*	0	-	40	*(35-45)*	Y	Y
36	ICSI	4	4.8	*(2.7-6.2)*	20.5	*(12-25)*	10	*(5-10)*	1	*(0-1)*	65	*(64-85)*	Y	Y
37	ICSI	6	2.5	*(1.5-2.8)*	2	*(1.3-3.1)*	18	*(5-25)*	1	*(0-2)*	80	-	Y	Y
38	ICSI	2	*6.4	*(5.5-7.3)*	3	*(0.2-3)*	33	*(30-35)*	1	*(0-2)*	70	-	Y	N
39	ICSI	1	4.5	-	3	-	15	-	0	-	35	-	Y	Y
40	ICSI	9	4.5	*(3.3-9.1)*	14	*(4-40)*	5	*(1-5)*	1	*(0-2)*	74	*(60-85)*	Y	N
41	ICSI	2	2.1	*(1.9-2.3)*	7.5	*(5-10)*	48	*(40-50)*	1	*(1-2)*	65	-	Y	Y
42	ICSI	1	1.7	*(1.5-1.9)*	3	*(3-3)*	15	*(15-15)*	2	*(1-3)*	65	-	Y	Y

Table 1 Semen analysis of partial globozoospermia patients.

Pat.: Patient couple number. Treatment: Fertility treatment. None; no treatment. IUI; Intra Uterine Insemination, Homologous. IVF; In Vitro Fertilization. ICSI; Intracytoplasmic Sperm Injection. 1ICSI, no ovum pick up; 2 poor fertilization in IVF and converted to ICSI in the next cycle; 3total fertilization failure in IVF and converted to ICSI in the next cycle. n: number of semen analyses. med.: median. * Volume including medium (HTF). Morphology (%); percentage of sperm cells with normal morphology. Acrosomeless (%); percentage of sperm cells without acrosomes. Incl.; included for analysis (Yes/ No). Preg.; Pregnancy (Yes/No). # noted as increased number of round-headed acrosomeless spermatozoa, without percentage.

n	Patients		Controls		*p*
	27		263		
	median	range	median	range	
Age women at first cycle	30.7	*(20.9- 37.3)*	30.7	*(21.3-37.5)*	0.94[a] (match)
Duration of fertility disorder (years)	3.7	*(1.0-8.9)*	3.5	*(0.7-14.4)*	0.54[a]
Duration of treatment (years)*	0.5	*(0.0-3.4)*	0.5	*(0.0-6.5)*	0.95[a]
ICSI cycles (n)	2	*(1-5)*	2	*(1-10)*	0.32[b]
Obtained oocytes per cycle (n)	9	*(1-21)*	11	*(1-30)*	0.27[a]
Fertilization rate per cycle (%)	75	*(20-100)*	75	*(14-100)*	0.86[a]
Transferred embryos per cycle(n)	1.8	*(0-2)*	1.8	*(0-2)*	0.78[b]
% A- embryo/ total transferred embryo's	67	*(0-100)*	47	*(0-100)*	0.13[a]
% B- embryo/ total transferred embryo's	21	*(0-100)*	32	*(0-100)*	0.16[a]
% C- embryo/ total transferred embryo's	12	*(0-100)*	21	*(0-100)*	0.66[a]
Cryopreservation (%)	14.8		9.5		0.35[b]

Table 2 Treatment characteristics of PG patient and control groups.

[a] A linear mixed model was used to test the difference between the PG group and the control group that accounts for the matched design (multilevel model).

[b] A conditional logistic regression model was used in case of dichotomous outcomes.

The p-value represents the statistical difference between the PG group and controls, using the multilevel model (a) or the conditional logistic regression. Note that the dependent variables; number of cycles (≤ 2 or >2) the number of transferred embryo's (≤2 or >2) were dichotomized to facilitate calculation.

* Duration of treatment was calculated by the date of the first and the last ovum pick up. In case of 1 ICSI-cycle this may be 0.

fertilization rate, percentage of transferred A, B, or C embryos, or ability to freeze spare embryos.

Primary and secondary outcomes measures are presented in Table 3. The live birth rate in the patient group was higher compared with the control group (66.7% versus 50.0%, respectively), but this difference did not reach statistical significance (Fisher exact test: P = .09). The secondary outcome measures were not significantly different, either: pregnancy rate 74.1% versus 57.6% (Fisher exact test: P = .10); miscarriage 14.3% versus 18.5% (Fisher exact test: P = .77); and twin pregnancy 14.3% versus 21.6% (Fisher exact test: P = .58). No stillborns occurred in the patient group, compared with one stillborn in a singleton pregnancy and three stillborns as part of a twin pregnancy in the control group.

n	Patients		Controls		*p*
	27		263		
	n	%	**n**	%	
Pregnant couples	20	74.1	152	57.6	0.10[b]
Pregnancies	21		162		
• Miscarriages	3	14.3	30	18.5	0.77[c]
• Singleton live births	15	71.4	96	59.3	0.24[c]
• Singleton stillborn	0	0	1	0.6	0.20 [c]
• Twin live births	3	14.3	35	21.6	0.58[c]
• One of twin stillborn	0	0	3	8.6	1.00[c]
Total live born	21		164		
Total live births	***18***	***66.7***	***132***	***50.2***	***0.09[b]***

Table 3 Outcome of the episode characteristics for partial globozoospermia and controls.

[b] p-value using conditional logistic regression
[c] p-value the Fisher's exact test.

Information on the sex, gestational age, birth weight, and congenital abnormalities of the newborns in the PG group were obtained for 17 out of 18 cases. In this group, 6 boys and 11 girls were born. Mean gestational age was 38 weeks 1 day. Mean birth weight was 2,993.6 grams (within 20th-50th percentiles). One case of mild aortic coarctation was reported. One child died in the neonatal period (day 10) because of sepsis in prematurity (28 weeks 1day).

DISCUSSION

In this study, we report on the efficacy and safety of ICSI as a treatment for infertility due to partial globozoospermia, of which disorder little is known to date. We show that this group of couples performed at least equally to a general ICSI population regarding the live birth rate. This is a reassuring finding for patients with partial globozoospermia.

We chose to describe all 42 PG patients that were identified in an 8-year period at our center, and subsequently we chose to perform a per-treatment analysis (possibly including several ICSI cycles) rather than an analysis per ICSI cycle. We did so because we believe that the combination of description and case-control analysis provides us with the ingredients for proper counseling of

patients suffering from partial globozoospermia about their chances of conceiving a child at the start of an ART treatment.

From these data, we can conclude that spontaneous pregnancies can occur in cases with normal to mild oligospermia in combination with a mild asthenozoospermia and teratozoospermia (according to World Health Organization criteria [14]). Whenever spermatozoa with acrosomes are present, spontaneous conception can occur but possibly with a longer time to pregnancy, as was observed in three patient couples. This suggests that PG may occur more often than expected in a proven fertile population. When, however, patients do not conceive spontaneously within a reasonable period of 1 year, or even via IUI when the semen parameters are acceptable to do so, ICSI seems to be the optimal treatment, because poor fertilization or even total fertilization failure appears to be a common result after IVF.

We could not find a significant difference in any of the investigated parameters (including fertilization rate, percentage of quality A embryos, percentage of cryopreserved embryos of good quality, pregnancy rate, percentage of miscarriages, and live birth rate) between the PG and control groups. In fact, these couples performed somewhat better on the last three items, though not significantly. In addition, the follow-up of 17 out of the 18 children born in the patient group was also reassuring. No aberrations regarding gestational age at time of birth or birth weight (which was within the 20^{th}–50^{th} percentile) were found. In one child out of the 18, a congenital disorder was observed, namely, an aortic coarctation, representing a prevalence of 5.6%. Compared with the odds ratio for birth defects after ICSI (between1.4% to 2.0% (15), this is a high figure. We should, however, take the small sample size of our group into consideration before drawing any conclusions. Obviously, future research should focus on the occurrence of birth defects in a larger group of partial globozoospermia patients. The data should then be linked to studies on the molecular aspects of partial globozoospermia regarding DNA damage and/or possible genomic anomalies in the sperm head.

In one case a neonatal death was observed. This death occurred because of sepsis in a premature infant on the tenth day after birth. It is not probable that this neonatal death was causally linked to ICSI in general or ICSI with semen derived from partial globozoospermia patient in particular.

The round-headed spermatozoa in partial globozoospermia share a lot of characteristics with those found in total globozoospermia (1). In total globozoospermia, a reduced ability to fertilize is described in about one-half of the cases reported, for which oocyte activation is applied in various reports (5–11, 16). Because a previous study from our group (1) demonstrated an increased occurrence of structural abnormalities in "normal" oval spermatozoa derived

from PG men, we anticipated that these "normal cells" that are picked for ICSI could have the impaired ability to fertilize similar as in total globozoospermia. Interestingly, fertilization via ICSI does not appear to be negatively affected by these abnormalities, but they may still play a role in spontaneous fertilization or fertilization via IVF, so further research on partial globozoospermia is still warranted regarding this aspect.

In reviewing our data, we can conclude that we are now better equipped to counsel subfertile couples with partial globozoospermia. First, their chance to conceive spontaneously is lower compared with the normal population, but this chance is by no means absent, provided that the other semen parameters are favorable. When enrolled in a fertility program, however, ICSI is the treatment of choice. Second, their chance of conceiving a child via ICSI seems to be at least similar to the general ICSI population. Finally, there appears to be no increased risk for a miscarriage. Unfortunately, we cannot yet counsel on the risk of congenital anomalies in this population, because our study group is too small to draw definite conclusions on this aspect of the treatment.

This study shows that that in compromised male fertility due to partial globozoospermia, ICSI is an adequate treatment to get pregnant, although spontaneous pregnancies cannot be excluded. Pending future research on the ICSI results in a larger study, including the prevalence of birth defects and the molecular aspects of the sperm heads in this population, these patients should be reassured that they have a good chance to father a healthy child.

Acknowledgments

Gwendolyn Woldringh is gratefully acknowledged for providing data from her database of ICSI children.

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Part 3

Genetics

Chapter 6

Homozygous mutation in *SPATA16* is associated with male infertility in human globozoospermia

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Am.J.Hum.Genet., 2007. 81(4): p. 813-820.

ABSTRACT

Globozoospermia is a rare (incidence 0.1% in male infertile patients) form of teratozoospermia, mainly characterized by round-headed spermatozoa that lack an acrosome. It originates from a disturbed spermiogenesis, which is expected to be induced by a genetic factor. Several family cases and recessive mouse models with the same phenotype support this expectation. In this study, we present a consanguineous family with three affected brothers, in whom we have identified a homozygous mutation in the spermatogenesis-specific gene *SPATA16*. This is the first example of a nonsyndromic male infertility condition in humans caused by an autosomal gene defect, and it could also mean that the identification of other partners like *SPATA16* could elucidate acrosome formation.

Approximately 15% of couples are confronted with the inability to conceive after 2 years of unprotected intercourse (1). In about half of these cases, infertility is due to the inability of the male partner to produce spermatozoa of sufficient number (oligozoospermia), adequate motility (asthenozoospermia), or normal morphology (teratozoo- spermia) or to combinations of these defects. Globozoospermia (MIM 102530) is a rare but severe teratozoospermia, characterized by ejaculates consisting completely of round-headed spermatozoa that lack an acrosome or, in partial globozoospermia, containing a variable proportion (20%-90%) of acrosomeless spermatozoa (2–4). Men that are affected with total globozoospermia are infertile, and even the application of intracytoplasmic sperm injection (ICSI) has met with disappointingly low success rates (2). Globozoospermia originates from a disturbed spermiogenesis, and, although the underlying cause is still unknown, a genetic contribution appears to be supported by several familial case reports (5–7) and by three recessive mouse models involving CSNK2A2 (MIM 115442), *HRB* (MIM 600862), and *GOPC* (MIM 606845) (8–10). However, no causative gene mutations have been identified in these orthologues or any other human genes to date (11,12). We describe a family with three affected brothers, in whom we have identified a homozygous mutation in the spermatogenesis-specific gene *SPATA16* (MIM 609856). To our knowledge, this is the first example of a nonsyndromic male infertility condition in humans caused by a single gene defect. We investigated an Ashkenazi Jewish family with six brothers (three affected and three healthy) and four sisters (fig. 1D) that was identified at the Centre for Reproductive Medicine of the Dutch-Speaking Brussels Free University. The three unaffected brothers fathered seven, six, and five children, respectively, but the three affected brothers were childless and presented with a fertility disorder due to oligoasthenoteratozoospermia, showing the characteristics of total globozoospermia, such as roundheadedness and acrosomelessness, as shown by acrosin (MIM 102480) staining in figure 1A. No known consanguinity was reported, although the family belonged to an isolated Jewish population. A normal karyotype and no Y-chromosome microdeletion were found. In two brothers, ICSI was performed, but fertilization was poor, and no pregnancy occurred.

We performed a genomewide scan analysis of all six brothers, using a 10K SNP array (Affymetrix GeneChip). Regions of homozygosity were defined by the presence of 125 consecutive homozygous SNPs. Large regions of homozygosity were observed in all six individuals (tables 1 and 2), indicating consanguinity in the second or third degree in the family. Therefore, we considered this family to be consanguineous and expected the pathology to be autosomal recessive. We identified a unique region of haplotypic identical homozygosity shared by all affected brothers, in which the healthy brothers were heterozygous. The smallest

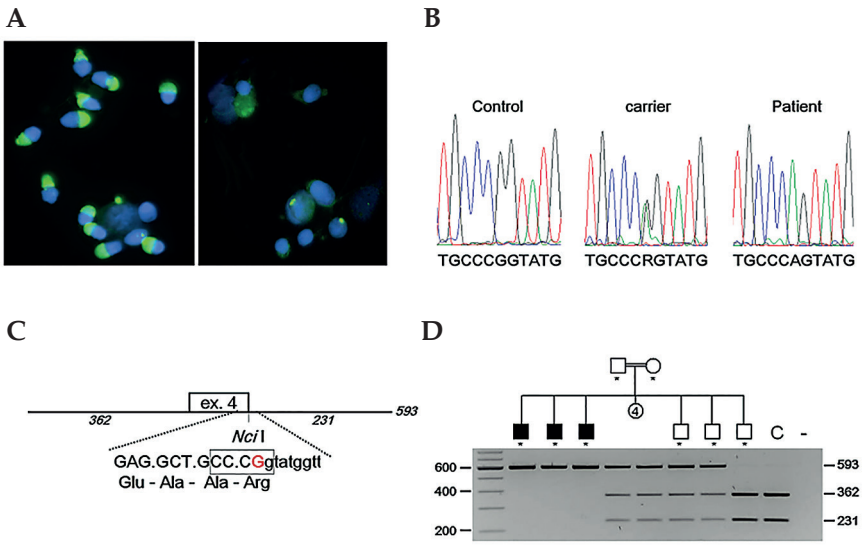

Figure 1 Family with globozoospermia and a mutation in the *SPATA16* gene.

A, Sperm morphology. Fluorescent acrosin (green) staining (with fluorescein) of acrosomes and 4,6-diamidino-2-phenylindole (blue) staining of nuclei. On the left is a sample from a fertile control, in which the most important content of the acrosome (acrosin) is clearly and abundantly present; on the right is a sample from a patient. Sperm morphology and acrosome structures are severely disrupted in patient cells. Remnants of acrosin staining were observed for some deformed sperm cells, but most signals represent nonspecific acrosin staining in the leukocytes. **B**, Chromatograms of the mutation. Shown are the sequences from a control sample, the heterozygous father, and one of the patients. **C**, The NciI recognition site (5-CCCGG-3) is lost because of the GrA mutation at the last nucleotide of exon 4. (The HpaII recognition site is not shown but overlaps at 5-CCGG-3). This mutation predicts a R283Q amino acid substitution, as well as the disruption of the 5 splice site of intron 4. **D**, Pedigree of the Ashkenazi Jewish family in this study. The order of the 10 siblings is arbitrary. The segregation of the mutation was studied by NciI digestion of a PCR amplification of exon 4 and its flanking sequences. The first lane is the marker lane. As asterisk (*) indicates tested individuals. The two parents and two siblings are heterozygous for the mutation. The three affected males are homozygous, and one unaffected male and a control (C) are not carriers of the mutation.

region of overlap spanned 17 Mb of chromosome 3q26 (167054711–184087390). This region contains 50 known genes in the UCSC Genome Browser. We selected the *SPATA16* gene (spermatogenesis-associated 16, also known as "NYD-SP12") as the most plausible candidate gene, because recent studies showed that *SPATA16* is specifically expressed in human testis and that the mouse ortholog is primarily expressed in the spermatocyte and spermatids (13). Localization in the Golgi apparatus and the shift with Golgi vesicles to the acrosome was observed in round and elongated spermatids by use of a SPATA19-GTP (green

Chr	Physical Position	SNP ID	S0389	S0390	S0391
3	165727121	SNP_A-1508753	AA	AA	AA
3	167054711	SNP_A-1512676	AB	AB	AB
3	167631594	SNP_A-1508627	AA	AA	AA
3	167801377	SNP_A-1519252	AA	AA	AA
3	169050879	SNP_A-1511126	BB	BB	BB
3	169140975	SNP_A-1509483	AA	AA	AA
3	169748882	SNP_A-1518417	BB	BB	BB
3	170222552	SNP_A-1518965	BB	BB	BB
3	170229669	SNP_A-1509719	BB	BB	BB
3	170266790	SNP_A-1519387	BB	BB	BB
3	170341708	SNP_A-1513150	BB	BB	BB
3	170440150	SNP_A-1513458	AA	AA	AA
3	170815817	SNP_A-1516975	AA	AA	AA
3	171062673	SNP_A-1514614	NoCall	NoCall	AA
3	171141972	SNP_A-1508020	BB	BB	BB
3	172368188	SNP_A-1510308	AA	AA	AA
3	172643136	SNP_A-1517656	BB	BB	BB
3	172771949	SNP_A-1509479	AA	AA	AA
3	172933454	SNP_A-1509435	AA	AA	AA
3	172933529	SNP_A-1509382	BB	BB	BB
3	173575998	SNP_A-1516388	BB	BB	BB
3	173580444	SNP_A-1514288	BB	BB	BB
3	173580729	SNP_A-1514241	AA	AA	AA
3	174671975	SNP_A-1507368	AA	AA	AA
3	175782878	SNP_A-1508795	BB	BB	BB
3	176537318	SNP_A-1511779	AA	AA	AA
3	176610712	SNP_A-1509801	BB	BB	BB
3	176827123	SNP_A-1511813	AA	AA	AA
3	177333023	SNP_A-1517824	AA	AA	AA
3	177621541	SNP_A-1518682	BB	BB	BB
3	177708137	SNP_A-1510834	AA	AA	AA
3	177723240	SNP_A-1516780	BB	BB	BB

Table 1 SNPdata of chromosome 3.

In this table, a part of the results of the SNP-array regarding the three affected brothers is presented. Several large area's of shared haplotype were identified, indicating consanguinity. The area of shared haplotype on which we concentrated is shown here. The homozygous region is hatched in grey.

Chr	Physical Position	SNP ID	S0389	S0390	S0391
3	178105620	SNP_A-1516425	AA	AA	AA
3	178105781	SNP_A-1515959	NoCall	NoCall	BB
3	179324469	SNP_A-1517000	NoCall	NoCall	AA
3	179597123	SNP_A-1516215	AA	AA	AA
3	179597352	SNP_A-1516746	AA	AA	AA
3	179636158	SNP_A-1512810	BB	BB	BB
3	179706441	SNP_A-1510950	BB	BB	BB
3	179839122	SNP_A-1514173	BB	BB	BB
3	180413909	SNP_A-1511671	AA	AA	AA
3	180729255	SNP_A-1508156	BB	BB	BB
3	181028753	SNP_A-1512457	BB	BB	BB
3	181180943	SNP_A-1507868	BB	BB	BB
3	181251555	SNP_A-1512336	AA	AA	AA
3	181298402	SNP_A-1513747	AA	AA	AA
3	181506449	SNP_A-1517808	BB	BB	BB
3	181552274	SNP_A-1509494	BB	BB	BB

Table 1 Continued.

fluorescent protein) fusion protein, strongly suggesting a role for the *SPATA16* protein in acrosome formation during spermiogenesis (14). *SPATA16* is composed of 11 exons encoding a highly conserved protein of 65 kDa (569 aa), which contains a tetratricopeptide repeat (TPR [MIM 602259]) domain. Sequence alignment (by use of ClustalW 1.81 [SDSC Biology Work-bench]) (fig. 2) shows that *SPATA16* is highly conserved across mammals, exhibiting an identity rate varying from 77% (mouse) to 96% (chimpanzee) (NCBI Blast) (table 3). The conservation is even higher (92% and 98% in mouse and chimpanzee, respectively) for the TPR domain, a protein-protein interaction domain commonly but exclusively found in cochaperone proteins (15). Sequence analysis of one of the affected sons revealed a homozygous sequence variation in exon 4 (c.848G>A), which disrupts a NciI or an HpaII recognition site (fig. 1C). Restriction-enzyme analysis revealed that the three affected brothers are homozygous and that the two parents and two healthy brothers are heterozygous for the mutation. The third unaffected brother appeared to be homozygous for the wild-type sequence (fig. 1D). The c.848G>A nucleotide variation is not known in any SNP database and was not identified in 231 controls, including 151 random controls of both sexes and 80 fertile males. The mutation predicts an amino acid change of a highly conserved

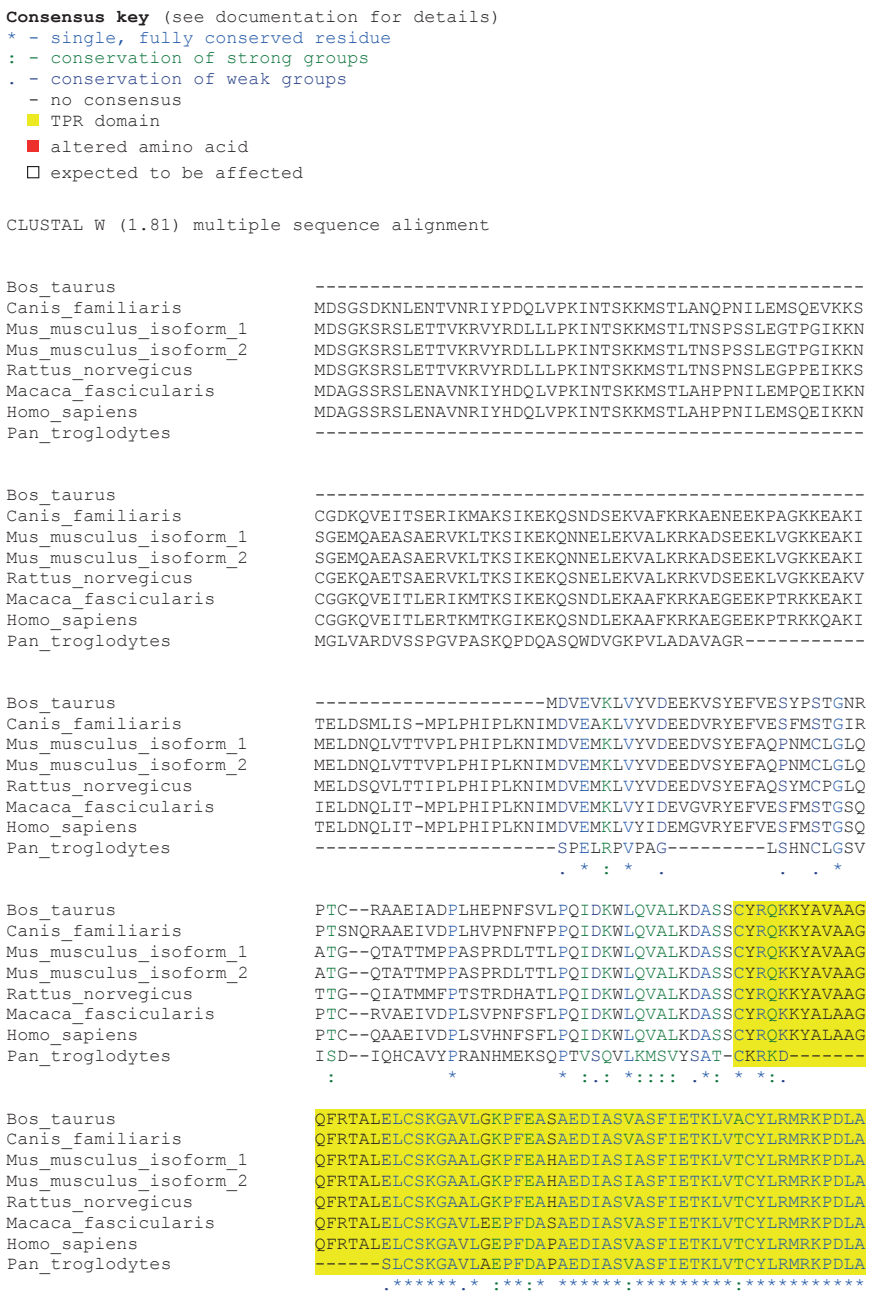

Figure 2 Sequence alignment SPATA16.

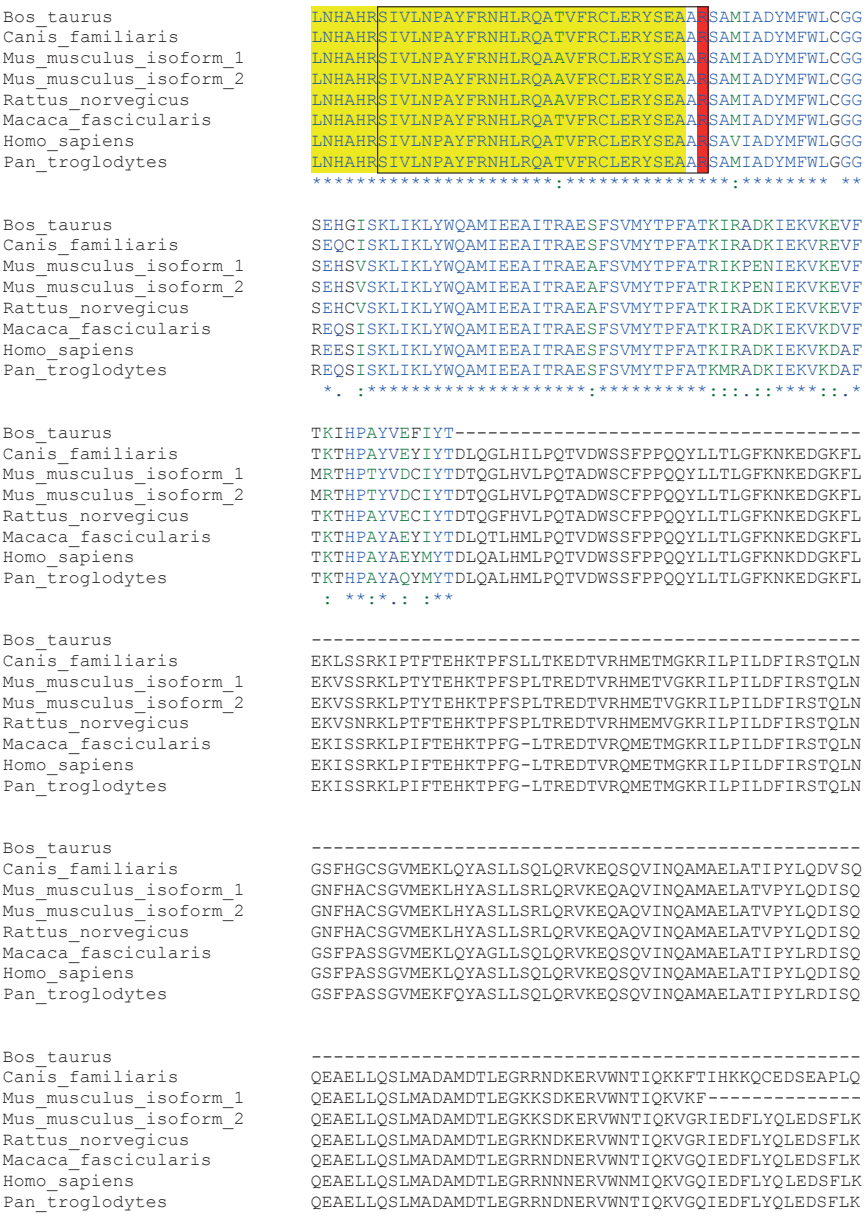

Figure 2 Continued.

```
Bos_taurus                  --------------------------------------------------
Canis_familiaris            RNKCKSPRTGWCRARGSSPATSCHGGKWSQRGEHLPVLLCCLPGHLENYC
Mus_musculus_isoform_1      --------------------------------------------------
Mus_musculus_isoform_2      TKKLRTARRQKTKMKRLQTVQQN---------------------------
Rattus_norvegicus           TKKLRTARRQKTKMKRLQTVQQN---------------------------
Macaca_fascicularis         TKKLRTARRQKTKMKRLQIVQQS---------------------------
Homo_sapiens                TKKLRTARRQKTKMKRLQTVQQR---------------------------
Pan_troglodytes             AKKLRTARRQKTKMKRLQTVHQS---------------------------

Bos_taurus                  -------
Canis_familiaris            CTNARKM
Mus_musculus_isoform_1      -------
Mus_musculus_isoform_2      -------
Rattus_norvegicus           -------
Macaca_fascicularis         -------
Homo_sapiens                -------
Pan_troglodytes             -------
```

Figure 2 Continued.

residue (p.R283Q) located at the C-terminal end of the highly conserved TPR domain. In addition, the c.848G>A mutation affects the last nucleotide of exon 4 (fig. 1C) and, therefore, may disrupt the 5 splice site of intron 4. Three different splice-site prediction models predicted that the mutation disrupts this splice site (table 4). Unfortunately, the *SPATA16* protein presents a testis- restricted expression, and we were not allowed to use fresh sperm cells or to perform a biopsy in these religious patients to verify the predicted aberrant splicing in vivo. Therefore, minigene constructs were made that consisted of two constitutive b-globin exons surrounding a 420-bp fragment containing either the wild-type or the mutated form of exon 4 and the flanking intronic sequences of *SPATA16*. These minigene constructs were transfected into COS1 or HeLa cells, and transcripts were analyzed by RT- PCR 24 h after the transfection (fig. 3A). As shown in figure 3B, wild-type exon 4 is invariably included in the final mRNA, as confirmed by the sequencing of the PCR product. In sharp contrast, the mutated exon gives rise to two aberrant splicing forms, as shown by cloning and sequencing of these PCR products. The most prominent, larger product is the result of the use of a splice site situated in the b-globin intron used for the minigene construct. The weaker, smaller product corresponds to the use of a cryptic splice site situated 18 bp upstream of the normal splice site. These aspecific products are likely the result of the very short intron sequences in the minigene construct. Such products are often seen in exon-trapping experiments in the absence of a bona fide splice site and are indicative of the occurrence of exon skipping due to the mutation (16,17) Importantly, we did not detect any transcript containing the correct junctions from the mutated exon 4, indicating that the mutation hinders normal splicing.

Chromosome and Start Position	Stop Position	Fragment Length (bp)	No. of SNPs[a]	Start Position	Stop Position	Fragment Length (bp)	No. of SNPs[a]
1:							
33955677	36556426	2,600,749	15				
117399167	119296010	1,896,843	11	117866019	119296010	1,429,991	6
151489481	156376556	4,887,075	11	151489481	156376556	4,887,075	11
160643582	162290883	1,647,301	14				
194965564	198362174	3,396,610	10				
2:							
19716714	34386124	14,669,410	55				
54483803	57217291	2,733,488	14				
59155900	65801963	6,646,063	11				
65801963	107509848	41,707,885	91				
113469814	115959977	2,490,163	12				
212719583	215032622	2,313,039	10				
224103332	226044921	1,941,589	16				
234216839	239151790	4,934,951	14	234719101	239151790	4,432,689	8
3:							
653347	3558063	2,904,716	12				
100634082	103786422	3,152,340	10				
113839242	127152417	13,313,175	48				
165655532	184087390	18,431,858	49	167054711	184087390	17,032,679	47
184087390	190712906	6,625,516	26				
4:							
173190930	176925273	3,734,343	10	173530324	176925273	3,394,949	8
181334851	191091333	9,756,482	48				
5:							
120723042	122283900	1,560,858	11	121110284	122283900	1,173,616	7
134671015	139500740	4,829,725	15				

6:								
	28766533	31094058	2,327,525	10	29479394	31094058	1,614,664	6
	46333713	47986997	1,653,284	14	46824038	47986997	1,162,959	5
	96879221	102207593	5,328,372	12				
	105174572	108446020	3,271,448	10				
	108446020	52383480	43,937,460	169				
7:								
	77627148	131407468	53,780,320	169				
8:								
	53838124	57959516	4,121,392	15				
	72728798	76200122	3,471,324	10				
9:								
	507715	13460671	12,952,956	85	12445236	13460671	1,015,435	6
	72760108	75532057	2,771,949	10	73265909	75532057	2,266,148	7
10:								
	63884288	66164664	2,280,376	10				
	66164664	68113302	1,948,638	10				
	91501439	105835217	14,333,778	47				
14:								
	19490525	22620727	3,130,202	20				
	31508337	33265230	1,756,893	10				
	36021565	37647284	1,625,719	10				

Table 2 Areas of Shared Haplotype and Shared Homozygosity.

NOTE – In the left part of the table, a selection of the areas of shared haplotype (those with (19 SNPs) is displayed. In the right part of the table, the regions of shared homozygosity (with 14 SNPs) that lie within are shown.

[a] Number of SNPs that form the area of shared haplotype or homozygosity.

Chromosome and Start Position	Stop Position	Fragment Length (bp)	No. of SNPs[a]	Start Position	Stop Position	Fragment Length (bp)	No. of SNPs[a]
15:							
21490270	23325412	1,835,142	13				
23325412	59077153	35,751,741	127				
79723090	84387340	4,664,250	12				
16:							
22705353	50026393	27,321,040	24				
17:							
28942222	34693022	5,750,800	10	29183029	34693022	5,509,993	9
66657008	72151125	5,494,117	10				
18:							
63746898	66906214	3,159,316	13	64766169	66906214	2,140,045	9
20:							
38790879	43185196	4,394,317	10				
21:							
18764912	21113081	2,348,169	11	18764912	21113081	2,348,169	11
29925652	31842275	1,916,623	12				
36234195	37974748	1,740,553	10	36447405	37974748	1,527,343	7

Table 2 Continued.

Species	Identity with Human (%)		Positives[a] (%)		Gaps[b] (%)	
	SPATA16	TPR Domain	*SPATA16*	TPR Domain	*SPATA16*	TPR Domain
Homo sapiens	100	100	100	100	0	0
Bos taurus	87	94	92	97	0	0
Canis familiaris	83	97	89	97	0	0
Macaca fascicularis	95	97	97	97	0	0
Mus musculus isoform 1	77	92	86	96	0	0
M. musculus isoform 2	78	92	87	96	0	0
Pan troglodytes	96	98	98	98	0	0
Rattus norvegicus	80	93	87	96	0	0

Table 3 Identity Rates among Species for the SPATA16 Sequence and the TPR Domain of the Protein.

[a] Amino acid positive-match score.

[b] Space introduced into an alignment to compensate for insertions and deletions in one sequence relative to another. In our search, the gaps did not exceed 0.5%.

Web Site[a]	Odds Ratio for Sequence	
	Wild Type	Mutant
NetGene2 Server	.80	<.50
SpliceSiteFinder	.805	.681
Splice Site Prediction by Neural Network	.97	<.40

Table 4 Splice-Site Predictions from Three Web Sites.

[a] See the Web Resources for URLs.

The first and critical step of exon inclusion is the binding of the U1 small nuclear ribonucleoprotein (SnRNP) splicing factor to the 5 splice sites (18). To confirm that the mutated *SPATA16* exon 4 is not recognized by the splicing machinery, we checked its binding to the U1 SnRNP by psoralen-mediated UV crosslinking. Whereas binding of U1 SnRNP to wild-type DNA was readily detected, this was not observed when DNA carrying the c.848G>A mutation was used as template (fig. 3C). The identity of the U1 SnRNP was confirmed by RNAse H treatment by use of an oligodeoxynucleotide complementary to

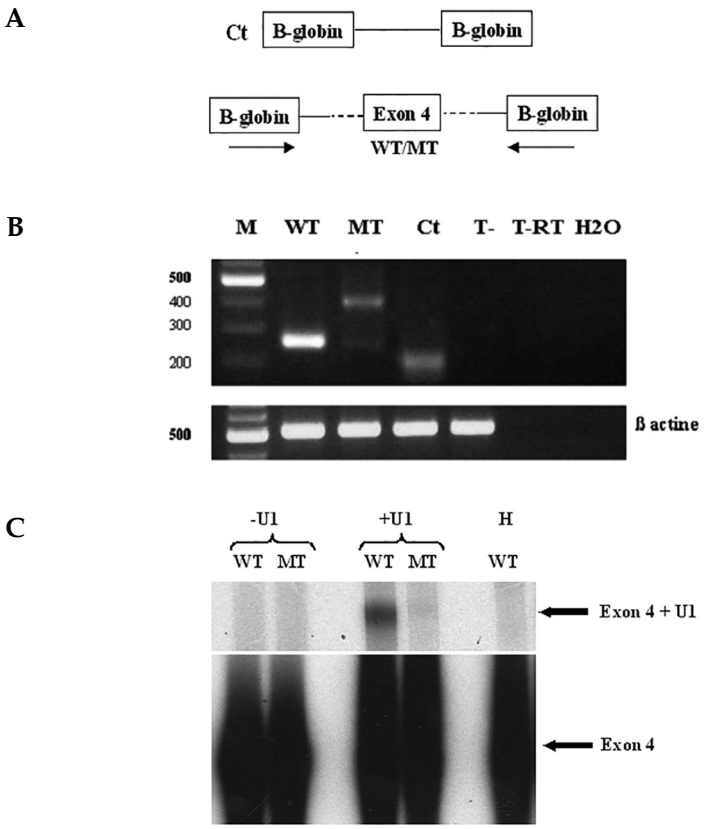

Figure 3 Mutated donor splice site of SPATA16 intron 4 and U1 SnRNP binding to wild-type (WT) and mutant (MT) donor splice sites.

A. Overview of the used prediction sites. **B**. Minigene constructs used to test the splicing of exon 4. T- = construct without exon; T-RT = construct without exon in RT-PCR. **C**. Gel showing that wild-type exon 4 is invariably included in the final mRNA. In sharp contrast, the mutant exon gives rise to two aberrant splicing forms. M = marker lane. **D**. U1 SnRNP binding analyzed by psoralen- mediated UV-crosslinking experiments, revealing that mutant exon 4 is not recognized by the splicing machinery, whereas wild-type exon 4 is clearly recognized. The identity of the U1 SnRNP was confirmed by RNAse H treatment with use of an oligodeoxynucleotide complementary to nucleotide positions 1–15 of U1 SnRNA.

positions 1–15 of U1 SnRNA (19). Therefore, the results of the of the bioinformatic prediction, the minigene, and the U1 binding strongly suggest that the c.848G>A mutation leads to inappropriate splicing of exon 4 and, therefore, the disruption of the TPR domain.

SPATA16 was also analyzed in 29 patients with globozoospermia, including 6 familial cases involving 14 patients. Of the 29 patients, 12 presented with total globozoospermia, and 17 with partial globozoospermia. None of them presented with any variation in the *SPATA16* sequence, with the exception of three known polymorphisms and two point mutations that did not segregate with the disease (table 5).

This is, to our knowledge, the first description of a gene involved in the pathogenesis of human globozoospermia. The data strongly suggest that the identified homozygous mutation in *SPATA16* causes globozoospermia in three of six brothers in the family studied, which allows us to state that globozoospermia can be a genetic trait with an autosomal recessive mode of transmission. The *SPATA16* protein localizes to the Golgi apparatus and to the proacrosomic vesicles that are transported to the acrosome in round and elongated spermatids during spermiogenesis. Our observations support the hypothesis of a crucial role for *SPATA16* in acrosome formation (14). The strongest protein conservation is seen in the TPR domain, which is disrupted in these cases of globozoospermia. The TPR domain is known to mediate protein-protein interactions and assembly of multiprotein complexes. Study of the x- ray structure revealed that the TPR domain adopts a helix- turn-helix arrangement, with the ability to associate with other a-helical structures. A possible interacting protein may be from the gene *GOPC*, a Golgi-associated protein containing coiled-coil motif a-helices(10), or from the HIV-1 rev binding protein gene (*HRB*), which also localizes to the Golgi complex (9). Both these genes are involved in the pathogenesis of globozoospermia in mouse models. Finally, it is worth noting that *SPATA16* contains six casein kinase II phosphorylation sites and that the casein kinase IIa is the most abundant casein kinase in the testis (8), for which the knockout model shows acrosome and other morphological defects (20). Moreover, the existence of several candidate genes (8–10) suggests genetic heterogeneity in human globozoospermia, which could be a reason why we did not find other patients with a gene alteration in *SPATA16*. Noteworthy as well is the fact that the heterozygous mouse models show no sperm abnormalities. This indicates that mutation carriers should have normal fertility. In this family, this seems to be the case, since the father and two heterozygous brothers have fathered 10, 7, and 6 children, respectively. In two of the affected brothers, ICSI was performed to induce fertilization and pregnancy, but without success. This is in accordance with the literature, which shows that ICSI enables oocyte fertilization, but with low fertilization rates in about half of the cases.

Since male infertility does not respect the canonical rule of genetics, the determination of inheritance patterns and the elucidation of genetic causes are complicated. Several genetic factors have been described that affect male fertility

Patient	Variations				Segregation		
	partial/ total	Exon	Variation	Known SNP?	Parents	Siblings	Ethnicity
1	partial	2	c.232G>A p.E78K	yes	-	-	European
			c.397A>G p.M133V	yes	-	-	European
2	partial	2	c.232G>A p.E78K	yes	-	-	European
			c.397A>G p.M133V	yes	-	-	European
		10	c.1526 C>T p.A509V	no	Mother	Absent in an affected brother	European
			c.1577 T>C p.M526T	no	Mother	Absent in an affected brother	European
3-4	partial	2	c.232G>A p.E78K	yes	-	-	European
			c.397A>G p.M133V	yes	-	-	European
5	partial	2	c.232G>A p.E78K	yes	-	Absent in an affected brother	North African
			c.397A>G p.M133V	yes	-		
			c.440G>A p.G147E	yes	-		
7-8	partial	2	c.232G>A p.E78K	yes	-	these patients are brothers	European
			c.397A>G p.M133V	yes	-		
			c.440G>A p.G147E	yes	-		
9	total	2	c.397A>G p.M133V	yes	-	-	European
10	total	2	c.232G>A p.E78K	yes	-	-	North African
			c.397A.G p.M133V	yes	-		
			c.440G>A p.G147E	yes	-		

Table 5 Polymorphisms.

In this table all non-synonymous, coding variations that were found in 9 out of 28 patients are shown. Patients 1 to 5 and 7 to 8 suffer from partial globozoospermia, whereas patients 9 to10 suffer from total globozoospermia. Three known SNP's were identified, next to two unknown, but non-segregating variations. In patients 6 and 11 to 28, no non-synonymous, coding variations were identified.

(21), but these give rise to more complex phenotypes. However, the patients in this study did not show any mental or physical abnormalities -in particular, no andrological abnormalities- in addition to their aberrant semen analysis. Thus, the mutation in *SPATA16* that we found in this study appears to present a human gene in which mutations give rise to male infertility without any associated other anomalies.

Further studies of other patients may help to identify other participant genes involved of the formation of the acrosome, allowing the fine dissection of the mechanisms involved in the setup of such a specialized cellular organelle. *SPATA16* defects influence spermiogenesis, whereas meiosis is not disturbed. Thus, modulation of *SPATA16* function or that of other components in the same pathway could offer an innovative, reversible approach to male contraception that is not based on controlling the hormonal pathway of sperm production.

Acknowledgments

We thank N. Dondaine, M. Jochem, J.-C. Nicod, and L. Ramos for precious technical assistance. We are grateful to the Institute of Genetics and Molecular and Cellular Biology Services and the Department of Human Genetics for their invaluable assistance. This work was supported by the French Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Ministère de l'Education Nationale, the l'Enseignement Supérieur et de la Recherche, the Louis Pasteur University of Strasbourg, and the Radboud University Nijmegen Medical Centre.

Web Resources

Accession numbers and URLs for data presented herein are as follows:

- BLAST, http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?
- dbSNP, http://www.ncbi.nlm.nih.gov/SNP/ (for exon 10 c.1526CrT [accession number ss73688634], exon 10 c.1577TrC [accession number ss73688636], and exon 4 c.848GrA [accession number ss73688635])
- NetGene2 Server http://www.cbs.dtu.dk/services/NetGene2/ Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for globozoospermia, CSNK2A2, HRB,
- GOPC, SPATA16, acrosin, and TPR)
- SDSC Biology Workbench, http://workbench.sdsc.edu/ (for Biology Workbench 3.2 and ClustalW1.81)
- SpliceSiteFinder, http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html
- Splice Site Prediction by Neural Network, http://www.fruitfly.org/seq_tools/splice.html
- UCSC Genome Browser, http://genome.cse.ucsc.edu/ (for March 2006 version)

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Chapter 7

General discussion

GENERAL DISCUSSION

In this thesis I have reported an extensive study on partial globozoospermia, comprising an analysis of the typical morphological aspects, the molecular characteristics and the clinical implications of this syndrome. Although I could not succeed in finding a causative genetic factor in a family with partial globozoospermia, I was successful in identifying the first human gene *SPATA16*, carrying causative mutations in complete globozoospermia.

The main findings of this thesis are:

- *Partial* globozoospermia is a heterogeneous sperm morphology disorder.
- *Partial* globozoospermia is best described as a heterogeneous sperm morphology disorder that consists of a high incidence of round-headed (>25%) and/ or acrosome-less sperm cells (>25%).
- The evaluation of sperm integrity in *partial* globozoospermia shows an early deacetylation defect, also in apparently normal shaped sperm cells. Although the integrity markers for protamination, chromatin condensation and DNA damage are augmented, still normal sperm can be found in PG.
- Spontaneous pregnancies are rare but possible in *partial* globozoospermia.
- ICSI is the first treatment of choice for a couple of which the man has *partial* globozoospermia.
- Based on our observations, there is no indication for increased risk for congenital anomalies for the offspring in *partial* globozoospermia.
- *Total* globozoospermia is a rare sperm morphology disorder, with a clear genetic background different from *partial* globozoospermia.

Morphology and integrity of *partial* globozoospermia

In chapter 3, I have studied the morphological aspects of partial globozoospermia. Our group established that the morphological features of partial globozoospermia differ from the normal heterogeneity in normozoospermia, as well as from the homogeneous total globozoospermia. Partial globozoospermia can be classified as an oligoasthenoteratozoospermia (OAT) that contains an increased amount of round-headed sperm cells (>25%) without acrosomes, as well as an increased occurrence of acrosome defects (>25%) next to other types of deformed spermatozoa.

Also with respect to acrosomal contents, partial globozoospermia appears to be grouped between normozoospermia and total globozoospermia. The morphological phenotype of partial globozoospermia shows a higher variability than already seen in human sperm. The morphological features of round-headed, acrosomeless cells in partial globozoospermia were, however, comparable to those in total

globozoospermia as examined by transmission electron microscopy (TEM; chapter 3). Round-headed sperm cells are accompanied by acrosome malformations and aberrant chromatin condensation. This malformation can be also sparsely found in the ejaculate of normozoospermic men.

While the study of chapter 3 was performed to describe partial globozoospermia, chapter 4 included the same samples to focus on molecular aspects of the sperm integrity, in order to investigate whether it is responsible to treat patients with this disorder to father their own genetic child. Apparently partial globozoospermia sperm cells tend to have a smaller head, the reason for the decreased surface area could not be explained as we would expect that poor condensed chromatin produce larger heads. The reassuring findings in chapter 4 is that still 'normal' sperm can be found and that the right sperm selection of sperm during ICSI will probably not affect clinical outcome (chapter 5).

Clinical features in partial and total globozoospermia

Despite the immunohistochemical abnormalities described in chapter 3 and 4, the partial globozoospermia couples performed at least equally well to a unselected ICSI population regarding the live birth rate after ICSI. Fertilisation rates in ICSI are not negatively affected in partial globozoospermia, probably because only 'normal (oval)' sperm is selected for injection. Finally, no increased risk for a miscarriage has been observed. In this population I did not find major congenital anomalies, but it is still too early to draw definitive conclusions because of the small study size. Altogether, these results appear reassuring for patients with partial globozoospermia. Although partial globozoospermia males may be occasionally accountable for spontaneous pregnancies, there is typically a long time to a pregnancy to occur. When patients do not conceive spontaneously within a reasonable period of one year, ICSI seems to be the optimal treatment. Since our paper in 2012 [1], no further studies on assisted reproductive techniques or pregnancy in partial globozoospermia have been published.

Men suffering from total globozoospermia can only father a child by ICSI. As described in chapter 2, the sperm of these males have an impaired capacity to fertilize even by ICSI. Since the publication of chapter 2 (2007), a total of 11 patients (case reports) have been presented that successfully achieved live births [2-9]. Again, in at least seven cases fertilisation needed to be induced with oocyte activation by either mechanical, electrical or calcium ionophore oocyte activation. Taylor (2010) describes that phospholipase C (PLC) expression, a recently established sperm factor involved in oocyte activation localised at the equatorial part of the sperm head, is absent in total globozoospermia as a cause for impaired oocyte activation [7, 10].

Genetic aetiology

We speculate that the origin of partial globozoospermia is an autosomal recessive genetic defect, as we identified a consanguineous family in which several members were diagnosed with *partial* globozoospermia. Unfortunately, we did not manage to find the underlying gene defect in this family (chapter 1). As we cannot exclude that another mode of inheritance underlies the condition in this family, we suggest to apply whole exome/ or genome sequencing for further genetic studies to resolve the genetic defect.

Our group and a French collaborating group succeeded to find a homozygous, autosomal recessive genetic mutation in the *SPATA16* gene in another consanguineous family in which three brothers suffered from *total* globozoospermia [11] (chapter 6). This was the first uncovered gene defect that caused *total* globozoospermia in humans. The *SPATA16* protein localizes in the Golgi apparatus and in the proacrosomic vesicles that are transported to the acrosome in round and elongated spermatids during spermiogenesis, implying a crucial role for *SPATA16* in acrosome formation [12]. The gene is disrupted in the highly conserved TPR domain that is known to mediate protein-protein interactions and assembly of multiprotein complexes.

Since our discovery, other genetic genes or mechanisms causing human globozoospermia have been revealed (Figure 1). First, a homozygous missense mutation in the *PICK1* (protein interacting with C kinase 1) gene was found in a member of a Chinese family with autosomal recessive globozoospermia [13]. This gene codes for a protein that is involved in protein trafficking and localized to Golgi-derived proacrosomal granules and was already described to cause globozoospermia in a knockout mouse model [14]. It is therefore the only gene to date reported to cause globozoospermia in both mice and men [13, 14].

Homozygous or compound heterozygous mutations in *DPY19L2*, located at on chromosome 12, appear to be a major cause for total globozoospermia [8, 15-19]. Deletions encompassing the *DPY19L2* gene arisen by non-allelic homologous recombination between the flanking low-copy repeats are most commonly seen in patients [17]. In total 93 out of 144 globozoospermia patients appeared to have a mutation within *DPY19L2*. The protein derived from *DPY19L2* is strongly expressed in the testis. Koscinski et al. (2011), examined the immature germ cells in the semen of three globozoospermic patients and concluded that in humans, *DPY19L2* is involved in the formation of the acrosome, but not directly on the acrosomic vesicles like *SPATA16* and *PICK1* [8]. Since then, the localisation of *DPY19L2* in spermatids was found to be restricted to the inner nuclear membrane facing the acrosomal vesicle [20]. The nuclear dense lamina (NDL) and the junction between the acroplaxome and the nuclear envelope is destabilised and, consequently, the acrosome and the manchette fail to be linked to the nucleus.

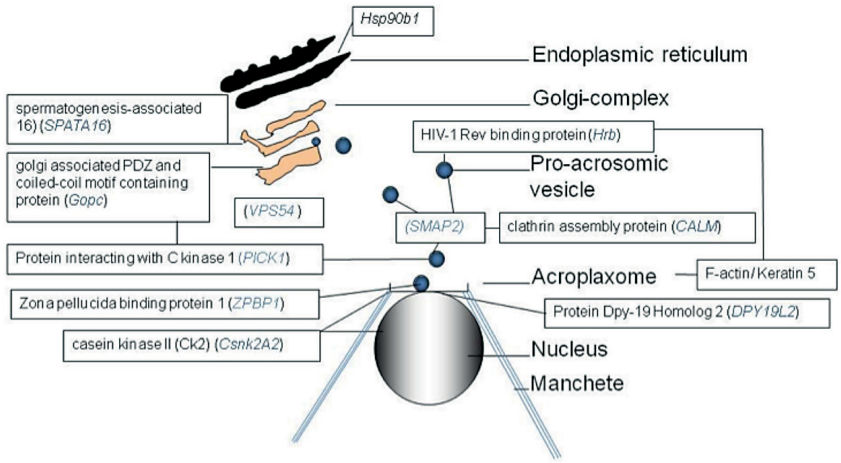

Figure 1 All known involved genes in total globozoospermia and their location during spermiogenesis.

This leads to the disruption of vesicular trafficking and probably failure of sperm nuclear shaping and the elimination of the unbound acrosomal vesicle by the discarding of the residual body. This is very reminiscent of the studies of Holstein et al. (1973), who showed that the acrosome is first formed in the free cytoplasm, and then discarded together with the residual body [21].

Next to these "globozoospermia genes" identified in humans, several other genes have been shown to cause a similar phenotype in mice (displayed in table 1). In chapter 6 we speculate about a possible interaction between *SPATA16* and other candidate genes that were identified as being expressed in the Golgi apparatus in mice, such as *GOPC* [22] or *Hrb* [23]. Subsequent research by other groups have provided support for this hypothesis. *PICK1* for example, was proven to interact with *GOPC* and the primary catalytic subunit of protein kinase 2 (CK2α'). The sperm acrosome associated protein 1 (SPACA1) is absent in *GOPC*$^{-/-}$ and *ZPBP1*$^{-/-}$ mice, suggesting a cascade of processes is needed to form the acrosome [24]. This is emphasised by another study, in which flagellum defects were described as a part of the defects next to anomalous development of Golgi-derived pro-acrosomic vesicles and failure of perinuclear theca proteins to properly associate with the nuclear surface [25]. The apprehension that each of these little steps can cause a similar phenotype, makes it logical that several descriptions of the pathogenesis of globozoospermia exist, simply because there are several components in the pathway that lead to similar abnormalities [26-36].

Therefore, we can conclude that *total* globozoospermia as a phenotype has various origins, as speculated earlier in chapter 2, in which *DPY19L2* plays a major role.

Name	Full name	Function	Reference
PICK1	protein interacting with C kinase 1	protein trafficking	[62]
Hsp90b1	heat shock protein 90 chaparone member	- protein folding - targeting of malfolded proteins to ER–associated degradation - calcium storage	[89]
SPACA1	sperm acrosome associated 1	component of the subacrosomal part of the perinuclear theca	[72]
SMAP2	small ArfGAP2	binds to the clathrin of the clathrin-coated proacrosomic vesicles and the clathrin assembly protein (CALM)	[91]
ZPBP1	Zona pellucida binding protein 1	in zona pellucida binding	[92]
Wobbler (WR)	Missence mutation in vesicular protein-sorting (VPS) factor 54	vesicular protein-sorting	[93]

Table 1 Mouse knockout models with phenotype globozoospermia.

FUTURE RESEARCH

Genetic background of partial globozoospermia

In chapter 4 we speculate that partial globozoospermia might originate from an epigenetic problem in spermiogenesis with a variable penetrance. Disruption of proper epigenetic signatures during the various phases in spermiogenesis will impact the tight transcriptional regulation of a plethora of genes [12]. It can be envisaged that already subtle deviations in expression of specific genes can cause a variable syndrome such as partial globozoospermia. The phenotype of the *Csnk2A2* knockout mouse, which was initially wrongly classified as total globozoospermia, seems to share certain traits with partial globozoospermia and might form a candidate gene[37, 38]. This gene is expressed in the brain and in the human testis. The *Csnk2A2* protein appears to promote cell survival and opposes cell death in the apoptotic pathways by phosphorylating several

proteins that induce caspase-mediated degradation during apoptosis [38]. We speculate that partial globozoospermia, as well other morphological defects, exists because the phosphor-labelled apoptotic spermatids escape degradation as suggested by Sakkas *et al* [39]. On the other hand, reduced protein levels or mutant proteins with residual activity could induce augmented levels of apoptosis during spermatogenesis, a possible reason why oligozoospermia is more common in partial than in total globozoospermia. Up till now no mutations in *Csnk2A2* have been found in globozoospermia patients [40] [41], but future research could consist of screening partial globozoospermia patients. A very interesting development in genetics using exome or whole genome sequencing may in the future help to find more genes involved in this highly complex process.

Can we restore fertility in globozoospermia?

As mentioned above, we can help partial or total globozoospermia patients by ICSI, with or without oocyte activation. Now that *DPY19L2* mutations are known to be the major cause of globozoospermia possibilities towards gene therapy could be explored, although ethical concerns regarding germ-cell therapy should be taken seriously. Recently, the creation of a *PICK1* transgenic mouse was published [42]. In this paper the authors infected *PICK1* knock-out mice per testicular microinjection with a lentivirus that incorporated *PICK1*. They did manage to induce expression of *PICK1* in knockout mice, which was still present after two semiferous cycles, suggesting that the new gene has been integrated into the genome. Unfortunately, they did not manage to restore fertility by directly microinjecting the knockout mice, probably due to the scarcity of infected spermatogonical stem cells (SSC) with a probable maximal overall ratio of infected SSCs of only 1.6%–6.4%. This could be tackled by increasing the viral titer and the combination of intratubular and intertubular injections. However, they did manage to create a transgenic line by infecting wild-type mice, who mated with the knockout mice. The male pups from this method appeared to be fertile due to the conserved PICK1-virus combination [42]. These genetic rescue experiments, which are still in its early developing stages, can provide proof-of-concept that rescue of infertility in globozoospermia is feasible in adult males. If so, this would raise hope that in the future patients with globozoospermia (or even other morphological abnormalities) could be treated with other strategies that would not make use of DNA integrating transgenic approaches. Finding additional causative genes for partial and total globozoospermia and further research into their molecular and cellular modes of action will be mandatory to develop targeted strategies towards correcting the effects of gene mutations.

Can globozoospermia play a role in the development of new contraception methods?

In chapter 6, we speculated on finding a way for male contraception that is not based on hormonal therapy [11], but by silencing *SPATA16*. We did not further investigate the opportunities to really do so. Another candidate gene in globozoospermia: *Hsp90b1* [43] could open other non-hormonal methods for male contraception. *Hsp90b1* was known to be involved in cancer and bipolar disorder; all members of the *Hsp90* family share an adenosine triphosphatase domain that can be blocked by anticancer drugs such as the derivatives of geldanamycin. The effect on the functions of the endoplasmic *Hsp90b* can affect spermatogenesis. Geldanamycin is however accompanied by a several severe other side effects, which make them unsuitable for contraception.

The uncovering of genes underlying male infertility may reveal a different view on how to reach an alternative to male contraception. Each of the known globozoospermia genes pictured in Figure 1, comprises its own perspective in the development of a non-hormonal contraceptive pill for men. By studying their exact molecular function and pathway in spermiogenesis, a strategy could be designed to try and develop an isolated and reversible effect in spermiogenesis that causes temporary sterility like acrosomelessness is and is safe to the future male fertility and offspring.

Final thoughts

So far, and probably due to the scarcity of cases presenting with this condition, it seems that after Holstein in 1973 we are one of the few worldwide that took the effort to collect data and investigate partial globozoospermia so far. We must consider that partial globozoospermia may have an important contribution to the frequency of failed fertilisation in IUI or IVF, but that it is not recognised as such during semen analysis and diagnosis. This condition has been named after his "big brother" total globozoospermia because of the augmented percentage of round-headed and acrosomeless sperm cells and we therefore speculated, like most others, that it originates from the same process that has been revealed in the recent years. However, this kind of teratozoospermia is not caused by the total absence of a protein but rather by the presence of a malfunctioning protein, as I have demonstrated in our studies presented in this thesis. Consequences as poor adhesion between the pro-acrosomic vesicles can occur. This might be tolerated in some cells, but other cells might be lacking an acrosome as a consequence. Also impaired repair can be a pathway considered. Multifactor processes should be expected and environmental factors cannot be ruled out [44].

Future research should focus on finding families with multiple cases of partial globozoospermia and screening them genetically. Protein arrays on

testicular tissue could help to identify which proteins are down or up-regulated in partial globozoospermia in comparison to fertile controls. Total globozoospermia has led to the revealing of several important steps in the spermiogenesis pathway, partial globozoospermia could be a suitable model to fill in the gaps.

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Chapter 8

Summary

SUMMARY

Chapter 1

This chapter comprises the general introduction of the thesis, with the backgrounds and motives for the studies that are performed in this thesis. The focus of this thesis is on the genetic background of (total) globozoospermia and the cellular, molecular, and clinical features of partial globozoospermia. We encountered this new sperm morphology disorder, in the search for a uniform phenotype to identify genetic factors underlying male subfertility. We described a consanguineous family with partial globozoospermia in an autosomal recessive pattern. Unfortunately, we could not identify the responsible gene for this morphological abnormality. Lastly, spermatogenesis and spermiogenesis are described as background information for this thesis.

Part 1

In part 1, all available literature on globozoospermia is assembled and summarised.

Chapter 2

This chapter consists of a review of all literature on globozoospermia until 2007. We conclude that globozoospermia is a rare (incidence <0.1%) disorder in male infertility. Total globozoospermia is diagnosed by the presence of 100% round-headed spermatozoa lacking an acrosome. Apart from the fact that affected males are infertile, no other physical characteristics can be associated with the syndrome. ICSI is a treatment option for these patients, although low fertilization rates after ICSI show a reduced ability to activate the oocyte. In globozoospermic cells, the use of acrosome markers has demonstrated an absent or severely malformed acrosome. Chromatin compaction appears to be disturbed but is not consistently over- or undercondensed. In some cases, an increased number of cells with DNA fragmentation have been observed. The analysis of the cytogenetic composition revealed an increased aneuploidy rate in some cases. Nonetheless, no increased number of spontaneous abortions or congenital defects has been reported in pregnancies conceived after ICSI. The pathogenesis of globozoospermia most probably originates during spermiogenesis, more specifically when the acrosome is formed and sperm head elongation starts. In several knockout mouse models, a phenotype similar to that in humans was found. These findings indicate a genetic origin, which is supported by the occurrence of affected siblings with globozoospermia in humans. We call for more research to elucidate the pathogenesis of human globozoospermia, but also to understand the steps in spermiogenesis and spermatogenesis in general. It is still unclear whether patients whose ejaculate contains both normal and

globozoospermic cells (partial globozoospermia) suffer from a variation of the same syndrome. For that reason, we conducted part 2 of this thesis.

Part 2

In part 2, partial globozoospermia is evaluated based on cellular, molecular, and clinical features.

Chapter 3

Partial globozoospermia is more commonly found during semen diagnostics than total globozoospermia. However, only one description on partially round-headed ejaculates has been published, dating from 1973. The aim of the investigation in this chapter was to describe partial globozoospermia compared to total globozoospermia and normozoospermia. Ejaculates from ten patients exceeding 50% of acrosomeless spermatozoa (partial globozoospermia), three patients with total globozoospermia, and nine normozoospermic controls were analysed using light microscopy, transmission electronmicroscopy, and flow cytometry. Qualitative and quantitative examination of spermatozoa from the three groups shows differences in the percentage of round-headed sperm cells and acrosome malformation. Total globozoospermia presents as a homogenous kind of teratozoospermia. Partial globozoospermia is a distinctive sperm malformation with an increased proportion (>25%) of round-headed sperm cells and acrosome malformations, that is different from total globozoospermia. In addition, ejaculates of males with partial globozoospermia contain oval sperm cells that have distinctive malformations of the sperm head matrix, but also morphologically normal sperm cells can be found which, based on pregnancy rates described for this group, are suitable to be used in a clinical setting (see chapter 5).

Chapter 4

In partial globozoospermia both the morphological 'normal' oval-headed as well as the round-headed shaped sperm show an increased percentage of structural abnormalities suggestive of an aberrant spermiogenesis. Therefore ten partial globozoospermia patients were evaluated in comparison to nine controls using the computerized karyometric image analysis (CKIA) for sperm head structure; fluorochrome Chromomycin A3 (CMA3) as a marker for protamination; TdT-UTP nick end-labelling (TUNEL) assay for DNA damage; testicular histone 2B (TH2B) as a marker for proper chromatin remodeling and KM-2 as an marker for disturbed acetylation and therefore a disturbed spermiogenesis and possible DNA damage. The percentage of normal spermatozoa for morphometry and densitometry was significantly lower for partial globozoospermia samples, an effect that persisted in oval sperm cells. No statistical

difference was found for CMA3 positive sperm, TUNEL positive cells or TH2B staining between controls an partial globozoospermia. KM-2 staining is significantly different in partial globozoospermia. When scored by morphology ('normal or abnormal' after decondensation), the differences for KM-2 are especially observed in the 'normal' group and disappear in the abnormal group. The conclusion from these data is that we still should be aware that in partial globozoospermia, spermatozoa might be abnormal, despite external morphological characteristics. In partial globozoospermia there are several indications that some processes during spermiogenesis are disturbed and that DNA damage is increased, although these aberrations do not differ from spermatozoa in normozoospermia.

Chapter 5

Between 1997 and 2005, 42 couples were identified in which the male presented with partial globozoospermia. In this chapter we describe the clinical outcome in these couples. Three spontaneous pregnancies were achieved with a longer time to pregnancy; two more pregnancies were achieved by IUI. Of the eight couples treated by IVF, seven had poor fertilisation and were referred to ICSI in the second cycle. In total, 27 couples had ICSI treatment and were matched with 263 control couples from a general ICSI population regarding female age and year of first ICSI cycle. One treatment, consisting of 1–10 ICSI cycles, was analysed. In the partial globozoospermia group, the live birth rate was comparable to the control group, as was the miscarriage rate. One major birth defect occurred, and one pregnancy ended in a neonatal death due to sepsis in a premature child, compared with four stillborn in the control group. We conclude that ICSI is an effective treatment in couples that failed to conceive spontaneously within one year combined with male infertility due to partial globozoospermia.

Part 3

In part 3, the first unveiled gene in human globozoospermia is described.

Chapter 6

In this chapter, we present a consanguineous family with three affected brothers, in whom we have identified a homozygous mutation in the spermatogenesis-specific gene *SPATA16. SPATA16* is specifically expressed in the testis and is known for its protein interaction. The *SPATA16* protein localizes to the Golgi apparatus and to the proacrosomic vesicles that are transported to the acrosome in round and elongated spermatids during spermiogenesis. Next to the fact that this is the first example of nonsyndromic male infertility condition in humans due to an autosomal gene defect, we speculate that the identification of other

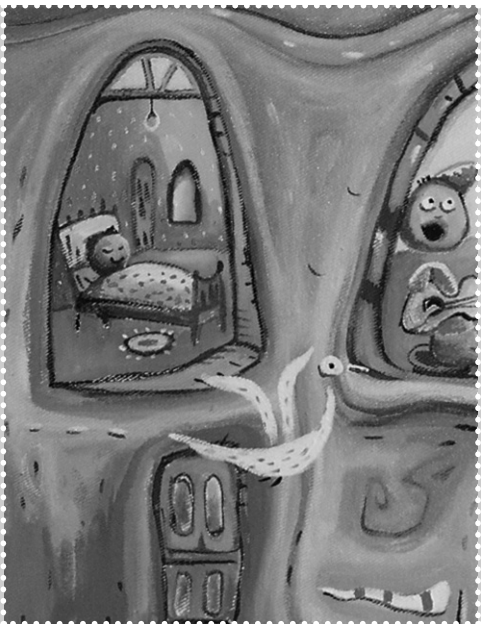
"globozoospermia genes" like *SPATA16* could elucidate acrosome formation and may therefore form an alternative path in male contraception.

Chapter 7

In the general discussion, the major conclusions are summarized and related to current literature. Newly revealed genes that are involved in the pathogenesis of globozoospermia are discussed, just as all new mouse models. We speculate on future research topics regarding partial globozoospermia and the unravelling of spermatogenesis.

Chapter 9

Samenvatting
PhD theses Human Reproduction NCEBP (2000 – 2014)
Dankwoord
Curriculum Vitae



SAMENVATTING

Hoofdstuk 1

Dit hoofdstuk bevat de algemene inleiding, met daarin de achtergronden en de aanleiding voor het onderzoek dat is uitgevoerd in het kader van dit proefschrift. Dit proefschrift gaat over op de genetische achtergrond van (totale) globozoöspermie en de cellulaire, moleculaire en klinische aspecten van partiële globozoöspermie. Partiële globozoöspermie werd ontdekt terwijl er gezocht werd naar een uniforme morfologische zaadafwijking om het onderzoek naar de genetische achtergrond van mannelijke subfertiliteit te kunnen voortzetten. Een consanguine familie, waarbinnen partiële globozoöspermie voorkwam in een autosomaal recessief patroon, wordt beschreven. Helaas kon het verantwoordelijke gen binnen deze familie niet gevonden worden. Het hoofdstuk wordt afgesloten met een algemene beschrijving van spermatogenese en spermiogenese, welke dient als achtergrondinformatie voor dit proefschrift.

Deel 1

In deel 1 is alle beschikbare literatuur over globozoöspermie verzameld en samengevat.

Hoofdstuk 2

Dit hoofdstuk bestaat uit een review van alle literatuur over globozoöspermie tot en met 2007. Hieruit concluderen we dat globozoöspermie een zeldzame aandoening is binnen mannelijke infertiliteit (incidentie <0.1%). Totale globozoöspermie kan worden beschreven als 100 % rondkoppige zaadcellen, waarvan het acrosoom mist. Er zij geen andere lichamelijke kenmerken geassocieerd met het syndroom, naast dat de aangedane mannen onvruchtbaar zijn. ICSI is op zich een behandeloptie voor deze patiënten, alhoewel er waarschijnlijk sprake is van een verlaagd vermogen tot oöcyt activatie gezien de lage fertilisatie cijfers. Met acrosoom merkstoffen is aangetoond dat het acrosoom geheel ontbreekt of minimaal sterk afwijkend is. Daarnaast lijkt chromatine condensatie verstoord te zijn, maar is er geen sprake van een consequente over- of ondercondensatie. In sommige beschreven casus werd een verhoogd aantal cellen met DNA fragmentatie gevonden. Ook werd in sommige casus een verhoogd aantal cellen met een aneuploïdie beschreven. Ondanks deze bevindingen wordt er geen verhoogd aantal miskramen of aangeboren afwijkingen beschreven bij zwangerschappen die uit ICSI ontstonden. Waarschijnlijk ontstaat het beeld van globozoöspermie tijdens de spermiogenese, in het bijzonder tijdens de vorming van het acrosoom wanneer de spermkop zich begint te verlengen. Bij diverse knock-out muismodellen werd een soortgelijk fenotype van globozoöspermie gevonden als bij mensen. Deze bevindingen wijzen in de richting van een genetische

oorsprong, wat wordt bevestigd door het vóórkomen van aangedane broers. We roepen op tot het doen van meer onderzoek om de ontstaanswijze van globozoöspermie bij mensen te verduidelijken, maar ook om de spermatogenese en spermiogenese in het algemeen beter te leren begrijpen. Op basis van het literatuuronderzoek is het echter nog steeds onduidelijk of partiële globozoöspermie een variatie is op totale globozoöspermie. Daarom werd deel 2 van dit proefschrift uitgevoerd.

Deel 2

In deel 2 wordt partiële globozoöspermie onderzocht op cellulair, moleculair, and klinisch niveau.

Hoofdstuk 3

Partiële globozoöspermie wordt vaker gediagnosticeerd bij routine semen analyse dan totale globozoöspermie. Er is echter maar één beschrijving over een gedeeltelijk rondkoppig ejaculaat uit 1973. Het doel van dit hoofdstuk is om partiële globozoöspermie te beschrijven, alsmede het te vergelijken met totale globozoöspermie en normozoöspermie. Daarvoor werden van 10 patiënten geselecteerd van wie de ejaculaten voor meer dan 50% bestonden uit acrosoomloze zaadcellen en vergeleken met 3 totale globozoöspermie patiënten en negen normozoöspermische controle patiënten door middel van lichtmicroscopie, transmissie electronenmicroscopie, en flow cytometrie. Kwalitatieve en quantitatieve bestudering van de zaadcellen van de 3 groepen laat verschillen zien in de percentages van rondkoppige zaadcellen en slecht gevormde acrosomen. Totale globozoöspermie presenteert zich als een homogene soort teratozoöspermie. Partiële globozoöspermie is een zaadafwijking met een verhoogd percentage van rondkoppige zaadcellen (>25%) en misvormingen van het acrosoom, wat anders is dan totale globozoöspermie. Daarbij komt dat het ejaculaat van deze mannen met partiële globozoöspermie ovale en dus normale vorm van zaadcellen bevatten die toch afwijkingen hebben van het zaadkopskelet, naast normale zaadcellen die, afgaand op de beschreven zwangerschapscijfers bij partiële globozoöspermie (Hoofdstuk 5), geschikt zijn voor een klinische setting.

Hoofdstuk 4

Bij partiële globozoöspermie worden zowel in normaal ogende zaadcellen als in de rondkoppige variant een verhoogd percentage van structurele afwijkingen gevonden die duiden op een verstoorde spermiogenese. Daarom werden 10 partiële globozoöspermie patiënten vergeleken met negen controles door middel van computerized karyometric image analysis (CKIA) om de zaadkopstructuur de evalueren; door middel van fluorochroom Chromomycine A3 (CMA3) als

indicator van protaminering; doormiddel van de TdT-UTP nick end-labelling (TUNEL) assay om DNA schade te meten; door middel van testiculair histon 2B (TH2B) als indicator van een goede chromatine remodelering en ten slotte door middel van KM-2 as indicator van een gestoorde acetylering en spermiogenese en mogelijke DNA schade. Hieruit bleek dat het percentage normale zaadcellen met betrekking op morfometrie en densitometrie lager was bij partiële globozoospermie, ook bij normaal ogende ovale zaadcelkoppen. Er werd geen significant verschil gevonden voor CMA-3 positieve zaadcellen, TUNEL positieve zaadcellen of TH2B kleuring tussen controles van partiële globozoöspermie. KM-2 kleuring bleek significant hoger bij partiële globozoöspermie. Als de scoring plaatsvond op onderscheid van morfologie (normaal of abnormaal na decondensatie), bleek het verschil voor KM-2 kleuring vooral te bestaan in de normale groep en te verdwijnen als de abnormale cellen werden vergeleken. We concluderen op basis van deze gegevens dat we ons altijd bewust moeten zijn dat ook normaal gevormde zaadcellen in geval van partiële globozoöspermie abnormaal kunnen zijn. Er zijn een aantal aanwijzingen dat sommige processen tijdens de spermiogenese gestoord zijn en op basis daarvan de DNA schade toegenomen, hoewel de uiteindelijke DNA schade niet significant verschilt van zaadcellen bij normozoöspermie.

Hoofdstuk 5

Tussen 1997 en 2005 werden 42 koppels geïdentificeerd, van wie de man aan partiële globozoöspermie bleek te lijden. In dit hoofdstuk beschrijven we de klinische zwangerschapsuitkomsten voor deze koppels. Er traden drie spontane zwangerschappen op, met een verlengde “time to pregnancy”; twee zwangerschappen werden tot stand gebracht door middel van IUI. Van de acht stellen die IVF ondergingen, hadden er zeven een laag bevruchtingspercentage en werden daarom verwezen voor ICSI bij de tweede cyclus. In totaal ondergingen 27 koppels een ICSI behandeling. Zij werden gematcht met 263 controle koppels uit een algemene ICSI populatie met betrekking tot de leeftijd van de vrouw en het jaar waarin de eerste ICSI cyclus plaatsvond. Eén behandeling, welke kon bestaan uit 1-10 ICSI cycli, werd geanalyseerd. In de partiële globozoöspermie groep was het percentage levendgeborenen en het aantal miskramen vergelijkbaar met de controle groep. Er was sprake van één ernstige aangeboren afwijking. Eén zwangerschap eindigde in neonatale sterfte vanwege een neonatale sepsis bij prematuriteit, vergeleken met vier perinatale sterftes in de controle groep. We concluderen dat ICSI een effectieve therapie is in het geval van partiële globozoöspermie, indien binnen 1 jaar geen spontane conceptie is opgetreden.

Deel 3

In deel 3 wordt de ontdekking van het eerste gen dat betrokken is bij humane globozoöspermie beschreven.

Hoofdstuk 6

In dit hoofdstuk presenteren we een consanguine familie waarvan 3 broers aangedaan zijn met totale globozoöspermie. Binnen deze familie konden we een homozygote mutatie aantonen in het gen *SPATA16*, wat specifiek tot uitdrukking komt tijdens de spermatogenese. *SPATA16* komt specifiek tot uitdrukking in de testis en speelt een belangrijke rol in eiwit interactie. Het *SPATA16* eiwit komt tot uitdrukking in het Golgiapparaat en de proacrosomale blaasjes welke naar het acrosoom van de spermatide getransporteerd worden tijdens de spermiogenese. Naast het feit dat dit het eerste geval is van mannelijke onvruchtbaarheid op basis van een autosomaal recessieve aandoening welke geen deel uitmaakt van een syndroom, is het identificeren van andere globozoöspermie genen zoals *SPATA16* waarschijnlijk een hele goede manier om de vorming van het acrosoom te ontrafelen en daarmee alternatieve wegen voor mannelijke anticonceptie.

Hoofdstuk 7

In de discussie worden de belangrijkste conclusies en in samenhang gebracht met de huidige literatuur. Nieuw ontdekte genen die betrokken zijn bij het ontstaan van globozoöspermie worden besproken, evenals de nieuwe muis modellen. We speculeren verder over eventuele toekomstige onderwerpen van onderzoek met betrekking tot globozoöspermie en het verder uitpluizen van de spermatogenese.

PHD THESES HUMAN REPRODUCTION NCEBP (2000 – 2014)

2000

1. 07-02-2000 *Els van der Molen*
   Disturbed homocysteine metabolism endothelial dysfunction and placental vasculopathy
2. 29-06-2000 *Willianne Nelen*
   Risk factors for recurrent early pregnancy loss. Hyperhomocysteinaemia, thrombophilia and impaired detoxification
3. 05-09-2000 *Ina Beerendonk*
   Sodium and ovarian hyperstimulation. Some clinical and psychological aspects
4. 04-12-2000 Anne-Marie van Cappellen van Walsum
   Cerebral metabolism of hypoxic fetal sheep by NMR spectroscopy
5. 18-12-2000 *Friso Delemarre*
   Vascular aspects of human pregnancy. Clinical studies on sodium restriction and angiotensin infusion

2001

6. 10-01-2001 *Way Yee Wong*
   Male factor subfertility. The impact of lifestyle and nutritional factors
7. 05-06-2001 *Petra Zusterzeel*
   Biotransformation enzymes and oxidative stress in preeclampsia
8. 05-10-2001 *Cathelijne van Heteren*
   Development of habituation and memory in the human fetus
9. 10-10-2001 *Michael Gaytant*
   Cytomegalovirus and herpes simplex virus infections in pregnancy

2002

10. 25-01-2002 *Ron van Golde*
    Male subfertility and genetics
11. 21-05-2002 *Tanja de Galan-Roosen*
    Perinatal Mortality

2003

12. 08-01-2003 *Maarten Raijmakers*
    Oxidative stress and detoxification in reproduction with emphasis on glutathione and preeclampsia
13. 18-2-2003 *Sabine de Weerd*
    Preconception counselling. Screening & periconceptional health
14. 22-4-2003 *Iris van Rooij*
    Etiology of orofacial clefts. Gene-environment interactions and folate
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    Emotional impact of unsuccessful fertility treatment in women

2004

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18. 24-11-2004 *Tanya Bisseling*
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2005

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2006

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23. 09-11-2006 *Jesper Smeenk*
    Stress and IVF. Clinical consequences

2007

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25. 01-11-2007 *Alwin Derijck*
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26. 01-11-2007 *Godfried van der Heijden*
    The transmission of chromatin and DNA lesions by sperm and their fate in de zygote (2)
27. 03-12-2007 *Kirsten Kluivers*
    On the measurement of recovery following hysterectomy
28. 10-12-2007 *Rene Kok*
    Proton Magnetic Resonance Spectroscopy of Human fetal brain

2008

29. 10-12-2008 *Trudie Gerrits*
    Clinical encounters: Dynamics of patient-centred practices in a Dutch fertility clinic
30. 12-12-2008 *Wouter Tuil*
    IVF and Internet

2009

31. 06-03-2009 *Ineke Krabbendam*
    Venous reserve capacity & autonomic function in formerly preeclamtic women

32. 03-09-2009 *Arno van Peperstraten*
Implementation of single embryo transfer
33. 07-10-2009 *Wilson Farid Abdo*
Parkinsonism: possible solutions to a diagnostic challenge.

2010

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Dynamic MR imaging in female pelvic floor disorders
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36. 21-04-2010 *Selma Mourad*
Improving fertility care: the role of guidelines, quality indicators and patients

2011

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Observational studies in reproductive medicine
38. 04-04-2011 *Marijn Brouwers*
Why foetal development of the male reproductive structures sometimes fails. An epidemiologic study on hypospadias and undescended testis with a focus on endocrine disruptors.
39. 22-06-2011 *Marian Spath*
Risk estimate for fragile X-associated primary ovarian insufficiency: Genetic, environmental and reproductive factors
40. 30-06-2011 *Inge van Empel*
Patient-centredness in fertility care
41. 18-11-2011 *Gwendolyn Woldringh*
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42. 17-06-2011 *Tiny de Boer*
Aspects of surgery for pelvic organ prolapse and its relation to overactive bladder symptoms

2012

43. 20-01-2012 *Esther Haagen*
Guidelines in IUI care. Implications for quality improvement
44. 17-02-2012 *Loes van der Zanden*
Aetiology of hypospadias. From genes to environment and back
45. 11-04-2012 *Reda Z Mahfouz*
Oxidative stress and apoptotic biomarkers in human semen
46. 06-06-2012 *Marleen van Gelder*
The role of medical and illicit drug use in the etiology of birth defects. Epidemiological studies and methodological considerations.
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A cytological exploration of human spermatogenesis in non-obstructive azoospermia patients: an analysis of variation

2013

52. 16-01-2013 *Jacqueline Pieters*
Incidental Findings of Sex Chromosomal Aneuploidies in Routine Prenatal Diagnostic Procedures
53. 04-09-2013 *Ellen Lensen*
Surgery for pelvic organs prolapse with emphasis on the anterior compartment
54. 16-12-2013 *Renne Gerritse*
Cryopreservation of an intact ovary.

2014

55. 23-01-2014 *Dana Huppelschoten*
Improving patient-centredness of fertility care.
56. 07-02-2014 *Tom van de Belt*
#HCSM Social media en wiki's in de gezondheidszorg
57. 19-02-2014 *Lucie Martijn*
Patient safety in primary midwifery care.
58. 25-02-2014 *Kim van Delft*
Levator ani muscle avulsion following childbirth.
59. 04-04-2014 *Karin Lammers*
Pelvic organ prolapse and a magnetic resonance imaging view on pubovisceral muscle avulsions.
57. 22-08-2014 *Joris van Drongelen*
Vascular adaptation to pregnancy and relaxin
58. 19-09-2014 *Yvette Geels*
Improved Identification of High Risk Endometrial Carcinoma
58. 23-09-2014 *Lobke Bastings*
Improving female fertility preservation care
59. 14-11-2014 *Kim Meeuwis*
Clinical studies on genital psoriasis and HPV-related lesions: a multidisciplinary approach

DANKWOORD

Het is net niet op de 11e van de 11e, maar er zijn wel 11 jaar verstreken sinds ik mijn eerste stappen deed binnen "het Radboud" in de brandende hitte. Elf jaar: dat is een mensenleven. Ondertussen zijn we een heleboel life events en nog meer (trein) kilometers verder. Daar horen in alle fases heel veel mensen bij die ik heel graag wil bedanken: ik heb getracht compleet te zijn en heb daarbij geen volgorde van belangrijkheid aangehouden.

Allereerst alle patiënten: het is nogal wat om je zaad in te leveren, en helemaal voor onderzoek. Het is een nogal intiem iets om te vragen. Jullie zijn niet allemaal even uitgebreid in dit proefschrift terecht gekomen, maar zonder jullie was dit allemaal in den beginne al niet gelukt. Mijn grote dank!

Professor Kremer, beste Jan; wat een traject hebben we gelopen vanaf het eerste weifelende begin. Wordt het wel een promotie? Het was voor mij en jou niet altijd makkelijk als vreemde eend in patientcentered en kwaliteitsland, maar jij zag altijd het positieve met een ware helicopterview. "Beter is de vijand van goed." Ik ben je dankbaar voor je ogenschijnlijk onuitputtelijke geduld en vertrouwen. Ik hoop dat ik hiermee waar heb gemaakt dat vertrouwen te verdienen. Dank!

Dr Liliana Ramos; beste Liliana, van het begin af aan ben jij betrokken geweest en dan ook echt mijn co-promotor. Je kennis, positieve en actieve instelling en opbouwend kritische houding vond ik zeer inspirerend. Je werklust is legendarisch. Dank voor al je hulp en begeleiding in het lab, je steun bij schrijven, het heerlijke theoretische brainstormen, en zo nu en dan gewoon een kletspraatje. Megadank!

Professor van Bokhoven, beste Hans, een oase van rust en weloverwogenheid, me mee begeleidend in de snel veranderende wereld van de genetica. Altijd weer enthousiast en een wezenlijk geduld. Dank!

Alle leden van het IVF-team, het oude Fertendo, het nieuwe VPG: "de kelder". Bij jullie heb ik geleerd wat het is om deel te zijn van een team: iedereen even belangrijk in zijn eigen rol en taak, en allemaal voor de patiënt. Dat heeft me gevormd als dokter en draag ik nog immer met me mee. Superdank voor het gunnen van mijn eigen plek bij jullie. Een speciale dank voor het fertiliteitslab, in het bijzonder Hans, Leonie en Hannie, voor hun bijdragen in het verzamelen, voorbewerken en beoordelen van patiëntenmateriaal. Ook voor de rest, die mij heeft geduld om mijn kleuringen en tellingen te kunnen doen op het lab bij verhuizingen en ontij.

Astrid Oudakker; ook wat om een dokter aan je slip te krijgen die echt geen verstand heeft van wat en hoe PCR. Van de eerste gels, de -80 en de radioactieve ruimte tot aan de SNP array: je hebt me altijd geholpen en begeleid. Tussen de bedrijven door hebben we heel wat gedeeld: mijn speciale dank voor je oor en steun.

De rest van het antropogeneticalab heeft mij als vreemde eend in de bijt altijd geduld en ook geholpen met het zoeken van monsters, zoeken naar de oorzaak van een PCR error, niet werkende DNTP-tjes en vervuild water. Uiteindelijk ook het uitvoeren van verdere analyses en testen, die uiteindelijk niet tot ontdekking hebben geleid en dus niet in dit boekje zijn beland. Zoveel vissen, zo veel werk, zo weinig vangst. Dank!

Eerst was er die plek in een stoffig kantoor, waar bierviltjes onder het bureau de ladenkast eronder lieten passen met Gwendolyn, Bea, Ineke, Esther en Wouter, om na de grote verhuizing vervolgens de kantoortuinbewoners van het eerste uur te worden. Samen met Angèle, Willianne, Arno, Wouter, Selma, Irene, Dennis, Charlotte, Sabine en natuurlijk Nel naast soms wat flexonderzoekers en studenten . Met eigen lief en leed pot en onderzoekersweekend. Wat een tijden waren dat! Menige taart (want publicatie) , koffie, lunchpauze en BBQ zijn er gepasseerd, naast alle organisatie voor de stukjes voor een bruiloft of promotie. Dank voor die geweldige tijd!

Dank aan (het lab van) Peter de Boer voor het ideeën-sparren en de wijsheid. Dank Godfried voor de gezellige lunches waarbij het over alles en niets ging en je kritische biologen blik.Moby Dick heeft het boekje toch gehaald, al is het dan niet als omslag.

Adrienne: dank voor je bijdrage aan hoofdstuk 5en voor je flexibiliteit met een artsonderzoeker net in opleiding.

Professeur Escalier: On s'est jamais croisé en persona, mais merci beaucoup à livrer sans hésiter l'anticorps de pro-acrosin, parti de chapitre 3.

Professor Tournaye: geweldige filmpjes van een Bar Mitswa en vooral geweldig materiaal. Zonder dat materiaal was hoofdstuk 6 er niet geweest. Dank u wel.

Björn, we hebben er veel werk ingestopt en ik heb dat - ondanks mijn moeite met je onnavolgbare Brugse accent- heel erg gewaardeerd. Helaas kwam dat voorbereidende werk er niet uit. Dank voor je samenwerking!

Professeur Viville, Stéphane, merci pour le coopération en chapitre 6. Je suis très heureuse que votre groupe à reussit de découvrir le gène le plus important en globozoospermia depuis. Je vous souhaite encore plus de même succès dans le futur.

Isabelle; on a beaucoup partager d'une distance et j'étais tres contente à te rencontrer à l'ESHRE à Stockholm. Merci pour la coopération et support et beaucoup de succès pour ton carrière à Strassbourg.

Dan de opleiding: woelige tijden waarin je jezelf zo vaak tegenkomt: in "ons Catrien" twee keer een geweldige tijd gehad met het team op de verloskamers, afdelingen, polikliniek en OK. Bij jullie op bezoek gaan voelt nog steeds een beetje als thuis op de koffie gaan. De gehele maatschap: dank voor het mij willen opleiden, soms goedschiks, danwel kwaadschiks. Ik hoor jullie nog steeds dagelijks in mijn achterhoofd....

Vakgroep van het Radboud: niet zo makkelijk, maar wat heb ik veel bij jullie geleerd over organisatie, het vak, maar vooral over mezelf. Het was thuiskomen na even weg geweest te zijn geweest en toch ook weer helemaal anders. Dank voor het me de ruimte geven om te kunnen groeien, die ruimte heb ik naar mijn beste kunnen ook echt trachten te benutten. De teams op de verloskamers, OK, poli en natuurlijk "de kelder": dank voor de heerlijke samenwerking.

Mentor van het eerste uur: Alex. Je hebt mijn tranen niet gedroogd, maar hebt me laten lachen door me uit, of was het toe te lachen. Koester die tissuedoos nog maar even. Het komt er echt nog wel van dat ik 'm in zijn setting kom bekijken in Rotjeknor!

Olivier: ik had je officieel benoemd als mentor, maar heb je weinig nodig gehad. Bedankt dat je het ruimhartig op je wilde nemen.

Minouche, je nam je taak heel serieus en dat heb ik heel erg gewaardeerd. Dank voor je steun bij vervelende tijdingen. Nu ben ik eindelijk ook klaar!

Opleidingsgenoten: ik heb getracht om jullie allemaal; ANIOS, AIOS, BAIOS, TAIO's op te zoeken en jullie van dit feestje op de hoogte te stellen. Wat een unieke tijd is die opleiding waarin zoveel gebeurt. Ik ben blij dat ik met jullie allemaal heb kunnen samenwerken en wil jullie heel erg danken voor de steun die ik heel duidelijk heb ervaren aan alle kanten. Voor de huidige AIOS: pas goed op jezelf en elkaar!

Beste maten van de maatschap gynaecologie VieCuri, of beter gezegd Dieuwke, Annemieke, Iris, Ella, Petra, Erik en Hans. Ik voelde me vanaf het eerste moment tijdens de sollicitatie enorm op mijn gemak bij jullie. Dank jullie wel voor de kansen en de ruimte die jullie me geven om de gynaecoloog te zijn en te worden, die ik wil zijn, met alle voor- en nadelen die ik meebreng. Ik mag zelfs hard lachen ;-). Zonder die ruimte had ik dit niet afgekregen.

Beste arts-assistenten gynaecologie, ofwel mijn puppen, chickies of pullekes: wat heerlijk dat ik met jullie mag werken en een deel mag hebben in jullie opleiding. Het is fantastisch om mensen te zien groeien en gelukkig doen jullie dat allemaal stuk voor stuk op je eigen manier. Ondertussen leer ik weer heel veel van jullie. Ik hoop nu alleen dat jullie niet met een geit/varken/koe/paard voor bij de kippen aan komen zetten...

Alle medewerkers van de afdeling gynaecologie en verloskunde VieCuri Venlo/ Venray: jullie hebben me hartelijk verwelkomd en hebben jullie medewerking verleend aan al diverse projecten om het voor de patiënt in VieCuri weer iets beter te maken. Ik ben heel blij dat ik met jullie mag werken. Dit jaar is het dit feestje; ik hoop dat volgend jaar augustus beter geschikt is om de verse traditie van het 'zomaar zomer feestje' voort te zetten.

Beste leden van Premier Curi; door jullie wordt het (sociale) leven in Noord-Limburg wat rooskleuriger. Samen kunnen we het verschil maken in ons ziekenhuis. Ik hoop dat we doorgaan t/m het punt dat we een motorclubje worden. Dank voor de toegevoegde waarde!

Fabienne; sommige dingen moet je af en toe eens zeggen en dit is daar de gelegenheid voor. Ik ben er superblij mee dat we al zo lang vriendinnen zijn. Ik hoop dat dat nog heel lang mag blijven! Jij bent er altijd als het er toe doet en accepteert me zoals ik op dat moment ben. Je pen heb ik bij me hoor, deze dag. Dank voor het hart onder de riem.

Marieke en Wieteke; niet voor niets zijn jullie mijn paranimfen. Als onnavolgbaar roosterteam en assistenten vertegenwoordigers stonden jullie je mannetje (m/v) en regelden de boel. Ik heb jullie beiden enorm leren waarderen in die opleidingstijd. Voor mij zijn jullie twee prachtige wereldwijven waar je in tijden van nood èn plezier op kan bouwen. Ik ben dan ook een zeer dankbaar en gelukkig mens om jullie vandaag naast me te hebben. Ennuh: die proefschriften van jullie komen ook vast nog wel.

Lieke, ondanks dat het je niet altijd helemaal meer duidelijk is wat er nu eigenlijk gebeurt, ben ik er dankbaar voor dat je er nog bij kunt zijn. Nu heb je nog zo'n gepromoveerd schoonkind. Bèr is opnieuw een groot gemis, maar heeft zijn eigen plekje in mijn achterhoofd op deze dag.

Broers: alweer zo'n academisch feestje van zuslief. Hierna is het wel een beetje klaar hoor! Fijn dat we nog steeds een echte familie vormen samen met mam, Christa en Martine en de kids. Laten we dat nog heel lang doen in goede en kwade dagen. Sebastiaan, Charlotte, Kyran en Djuna: ik ben er heel trots op dat ik jullie Tantan mag zijn. En na de ceremonie kunnen jullie gewoon meefeesten! Mam: jij en pa hebben altijd gezegd dat je wel hersenen gehad kunt hebben, maar dat het om gaat wat je er mee doet. En dat dat een verantwoordelijkheid met zich meebrengt. Nou, dat heb ik geweten. Maar nu is het boekje af! Dank voor de basis die jullie altijd gevormd hebben,en de wijze woorden nu en dan. Pap: je snapte heel goed hoe belangrijk dat praatje was. Om daar nou precies op tijd voor te overlijden was ook weer wat overdreven. Maar ik ben gegaan en ik heb het gedaan. Ik vind het jammer dat je er niet lijfelijk bij bent, maar je bent van harte welkom in elke andere hoedanigheid!

Raoul, sjattie petattie. We gaan al heel wat jaren samen over de woeste baren van het leven, maar niks was zo veeleisend als dit traject en dan ook nog naast de opleiding. Je wilt nog niet eens dat ik je bedank in dat verdomde boekje, maar aan wie heb ik meer te danken dan aan jou? Je bent die heerlijke lastige man die enorm voor me zorgt. Nu is het boekje dan af; eindelijk! Ook al zal jouw pup altijd een werkbij blijven, zonder jou zou dat slecht lukken. Jij maakt mijn leven mooier.
Dikke poene: geniet ervan: dit is ook jouw feestje!

CURRICULUM VITAE

Anika Dam werd op 16 mei 1976 geboren in Roosendaal en Nispen. Zij groeide op in Bergen op Zoom, waar ze in 1995 haar eindexamen Gymnasium behaalde aan het RK Gymnasium Juvenaat H. Hart (tegenwoordige Gymnasium Juvenaat). Zij werd direct ingeloot voor de studie geneeskunde in Maastricht. Tijdens deze studie deed ze een keuzestage kindergeneeskunde in Dapaong, Togo; een wetenschapsstage psychiatrie bij de Université de Nantes in Nantes, Frankrijk en een wetenschappelijke keuzestage bij John Challis' lab bij Toronto University in Toronto, Canada. Ze was 2 jaar betrokken bij de studententoneelvereniging "Alles is drama" en als secretaris van de organisatie van het 4th Maastricht Medical Students Research Conference in 2000.

Ze behaalde in 2002 haar artsexamen en begon als arts-assistent niet in opleiding op de verloskamers van het Bernhoven ziekenhuis, locatie Oss en ging in Den Bosch wonen. In september 2003 zette ze haar eerste stappen binnen UMC St Radboud (het huidige Radboudumc) als IVF-arts en artsonderzoeker binnen het project "Morfologische aspecten en genotypering bij globozoö-spermie". Dit project leidde uiteindelijk tot dit proefschrift. In juli 2007 mocht ze beginnen met de opleiding gynaecologie binnen het Catharina ziekenhuis te Eindhoven (opleider: Tom Hasaart) en het UMC St Radboud (opleider: Didi Braat). Sinds januari 2013 is zij werkzaam als gynaecoloog binnen het VieCuri Medisch Centrum (locaties Venlo en Venray). Zij is sinds 2005 getrouwd met Raoul Martin.

"However, a good laugh is a mighty good thing, and rather too scarce a good thing; the more's the pity. So, if any one man, in his own proper person, afford stuff for a good joke to anybody, let him not be backward, but let him cheerfully allow himself to spend and to be spent in that way. And the man that has anything bountifully laughable about him, be sure there is more in that man than you perhaps think for."

Herman Melville, Moby-Dick; or, The Whale(1851)